Vol XXXI, No 1 *May, 1943*

THE INDIAN JOURNAL OF MEDICAL RESEARCH

PUBLISHED FOR
THE INDIAN RESEARCH FUND ASSOCIATION
BY
THACKER SPINK & CO (1933), LTD., CALCUTTA

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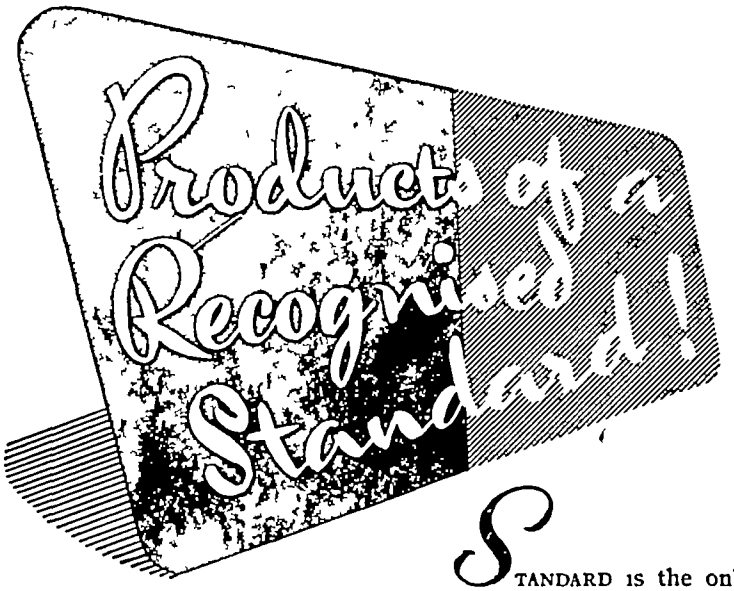

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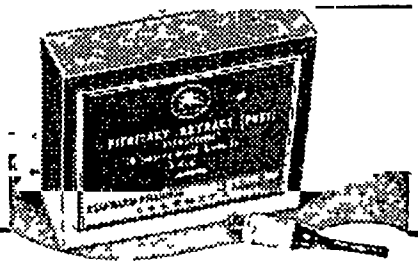

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CONTENTS

COLIFORM ORGANISMS FROM THE URINARY TRACT

BY

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AND

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[Received for publication, October 6, 1942]

THERE has, so far as we are aware, been no attempt made in India at classifying the *coliform* bacteria isolated from cases of urinary infection The results of preliminary studies carried out on seventy-two strains isolated from catheterized specimens of urine have, therefore, been presented in this paper The specimens were derived from patients in the Government Hospital for Women and Children and the Victoria Caste and Gosha Hospital Madras, suspected to be suffering from ailments of the urinary tract

TECHNIQUE

The specimens were plated out immediately on receipt on MacConkey agar plates and incubated for 24 hours at 37°C at the end of which single lactose-fermenting colonies were subcultured on agar slopes These colonies were studied for their morphology and staining reactions and then passed through the usual biochemical tests used in the study of *coliform* organisms They were also tested for motility and for capsule-formation Late lactose-fermentation was also tested for The Voges-Proskauer test was carried out using α-naphthol (Barrett's method) The results are given in Table I —

TABLE I

Koser	V P	M R	Indol	Number	Percentage	
+	+	−	−	21	29 2	*B aerogenes* includes *B cloacae*
−	−	+	+	25	34 72	*B coli*
+	0	0	−	1	1 4	*Intermediate?* or *B friedlanderi*
+	0	−	−	2	2 8	" " " "
+	−	+	+	7	9 7	" " " "
+	+	+	+	1	1 4	*B oxytocus*
−	−	−	−	2	2 8	Unclassified
−	−	−	+	2	2 8	"
−	+	−	−	2	2 8	"
+	−	−	−	3	4 2	*Intermediate?* or *B friedlanderi*
−	−	+	−	2	2 8	Unclassified
−	0	+	−	3	4 2	"
−	0	−	−	1	1 4	"
				72	100 2	

0 indicates that the test was not done

Only those which were positive to the Koser's citrate and the V-P tests and definitely negative to the M-R and Indol tests were grouped as *aerogenes* Our *B coli* group included those which were definitely M-R and Indol positive and negative to the V-P and Koser tests Our intermediates were grouped after the scheme of Malcolm (1938) except for the omission in our tests of inosite-fermentation 16·8 per cent remained unclassified as they did not conform to any of the standard groupings above cited

The results of our preliminary study on a comparatively small number of organisms would appear to be in agreement with those of other workers in England and America Hill *et al* (1929) found the range of *aerogenes* in human fæces to be from 0 06 to 16 per cent This range has been considerably exceeded in the case of urinary strains Hill *et al* (*loc cit*)

consider that this relative increase of *ærogenes* over the typical *coli* may be due to the greater powers of resistance exhibited by the *ærogenes* in *abnormal* situations such as the urinary tract Burke-Gaffney (1932) supports this view The preponderance of *ærogenes* in soil has been found to be due to a similar state of affairs

The application of the Eijkman test to the *coliform* organism in our study did not yield consistent results and showed the presence in our series of *ærogenes* which failed to grow at the higher temperature (44°C) The tests for motility and capsule-formation showed several discrepancies and failed to help in classification Similarly, the fermentation of carbohydrates yielded in some cases results which were neither consistent nor helpful in assigning the organisms to their proper groups Several *ærogenes* organisms grouped by the V-P and M-R and Koser and Indol reactions failed to ferment saccharose and some of the so-called *coli* organisms did not ferment dulcite

Discussion

Bacterial infections of the urinary tract are believed in many cases to have their source in the intestines (Usland, 1922, Walker, 1930, Nabarro, 1930) One would, therefore, expect the same proportion of the *coli* and *ærogenes* groups in fæces as well as in urinary infections, but, in actual experience, it has been found that in urinary infection, the ratio of *ærogenes* to *coli* is very much higher than what obtains in the large intestines

In practically all cases of intestinal disorders, e g enteritis, dysentery, typhoid, cholera, etc, we find that there is a definite variation bordering in some cases on a total upset of the normal *ærogenes/coli* ratio Krishnan and Chawla (1941) found that in cases of cholera, the *ærogenes* type in fæces may be as high as 95 per cent of the total *coliform* content Burke-Gaffney (*loc cit*) and Raghavachari and Iyer (1940) suggest that *B coli* loses its fæcal characteristics on being removed from its normal habitat, viz the large intestines

Table II

	Total	Percentage of						
		B ærogenes	*B coli*	*B oxytocus*	*Intermediates*	Atypical	Miscellaneous	Unclassified
Seshadrinathan and Venkataswami (1943)	72	29 2	34 72	1 4	18 1			16 8
Hill *et al* (1929)	1,000	39 5	50 0				10 5	
Burke Gaffney (1932)		52 0	33 0		10 0	5 0		

It would not be unreasonable now to argue that the changes occurring in the *ærogenes/coli* ratio may be and probably are due to a suppression of one type at the expense of the other, as a result of changes brought about by environmental conditions, thus, for instance, the alkaline pH of the content of the small intestines and the presence there of digestive ferments inimical to the life of the more susceptible group *B coli*, the acidic pH of the content of the large intestines, the possible absence of inimical ferments in that situation and the consequent favourable conditions for the growth and multiplication of *B coli*, these are indeed factors which appear to govern the *ærogenes/coli* ratio in the two situations In cases of diarrhœa, dysentery and cholera there will often be a constant tendency to hasten and rush the passage of the intestinal contents towards the rectum, and the environmental condition in the large intestines may, under such circumstances, approximate those found in the small intestines The relative preponderance there of *ærogenes* in the large intestines is easily explained A disordered state of the intestinal tract is found to be present in nearly all cases of urinary fection and the relative frequency and preponderance of the *ærogenes* types of organisms in uch infections can, therefore be easily accounted for

The preponderance of *ærogenes* in soil has been shown by many observers to be due to the greater powers of resistance of these types to natural forces at work and the favourable environmental conditions found in the soil, similar probably to those found in the intestines in the conditions noted above

Experiments to determine the effect of variations in the pH and of other environmental factors on the *coli/ærogenes* relation are being conducted

Summary

A study of 72 strains of *coliform* bacteria isolated from infections of the urinary tract showed that typical *ærogenes* forms were present in about 30 per cent, and typical *coli* organisms in about 35 per cent of cases *Intermediates* accounted for 18 per cent, while the rest could not be grouped This finding is in agreement with that of other workers in England and America

Acknowledgments

Our thanks are due to Rao Sahib T N S Raghavachari for the results obtained with modified Eijkman's test, and to Dr C G Pandit, M B , B S , Ph D , D P H , D T M , Director, King Institute, Guindy, for permission to publish this paper

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THE ACTION OF DYES ON VIBRIOS

BY

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ALTHOUGH the bacteriostatic action of dyes on certain micro-organisms has been the subject of a considerable amount of study (Kline, 1935 , McCulloch, 1936) we have been unable to trace any published records of their action on vibrios

The results of tests of bacteriostatic and bactericidal action of 22 selected dyes on *V choleræ* and vibrios of other serological types isolated from water or cholera cases are now presented The dyes tested were —

Brilliant green, crystalline (Grubler & Co)
Malachite green (Grubler & Co)
Gentian violet (Grubler & Co)
Crystal violet (Grubler & Co)
Safranin (Grubler & Co)
Methyl violet (Grubler & Co)
Basic fuchsin (Grubler & Co)
Acriflavin (Boots)
Thionin (Halborn & Sohn)
Eosin (Baird & Tatlock)
Methyl red (Merck)
Methylene blue (Merck)
Neutral red (Grubler & Co)
Mercurochrome (Grubler & Co)
Indigo carmine (Harleco)
Alizarin (Harleco)
Nigrosin (Harleco)
Aniline blue (Harleco)
Borax carmine (Harleco)
Pyronin yellowish (Harleco)
Fluorescein (Harleco)
Methylene violet (Harleco)

Preparation of dyes —Solutions were made in sterilized glass-distilled water at pH 6 8 and put in the steamer for half an hour and tested for sterility They were freshly prepared before use The dyes were found sterile by this method

Tests of bacteriostatic properties —Plates were prepared with nutrient agar (pH 7 0) mixed with different concentrations of dyes and incubated overnight before inoculation The inoculum consisted of purified young cultures of freshly isolated vibrios in peptone-water Plates were divided into four quadrants and a thick large loopful of one strain was spread in each of the quadrants by parallel linear strokes When the inoculum had dried, the plates were inverted and incubated at 37°C for 24 to 48 hours and sometimes more An absence of bacteriostatic effect was noted by observing confluent and discrete colonies on the plates When no colonies or only one small colony were found, this was regarded for practical purposes as indicating a complete bacteriostatic effect Only one or two strains were employed in these experiments on bacteriostasis, but 6 to 19 strains of each of the common species of vibrios were tested with methylene violet, pyronin yellowish and fluorescein Most of the experiments were repeated for confirmation

Tests of bactericidal properties —Quantities of 9 c c of peptone-water at pH 7 0 were inoculated with young cultures of vibrios and after incubation for 18 to 20 hours at 37°C were mixed with 1 c c of dye solutions and incubated for a further 2 to 20 hours The presence or discharge of the dye colour was noted A large loopful was then taken from the top of each tube, spread on an agar slope and incubated for 24 to 48 hours If there was no growth on agar $\frac{1}{2}$ c c was taken out and inoculated into about 7 c c to 8 c c of peptone-water to confirm its sterility As a rule it was found that when the colour of a dye persisted and turbidity in the medium did not increase the culture was found dead In later experiments, the practice of inoculating $\frac{1}{2}$ c c for testing sterility was abandoned, and further inoculations with loopfuls only were made if there was any suspicion regarding the full bactericidal effect In

TABLE

Bacteriostatic

	1—5,000								1—10,000							
	I	O	E	B	C	T	A	F	I	O	E	B	C	T	A	F
Brilliant green	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Crystal violet	−	−	−	−	−	+	+	−	−	−	−	−	+	+	+	+
Malachite green	−	−	−	−	−	+	+	−	−	−	−	−	−	+	+	−
Acriflavin	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+	−
Gentian violet	−	−	−	−	−	+	+	−	−	−	−	−	−	+	+	−
Thionin	−	−	−	−	+	+	+	±	−	−	−	−	+	+	+	±
Fuchsin	−	−	−	−	+	+	+	−	+	+	+	+	+	+	+	−
Methylene blue	−	−	−	−					−	−	−	−				
Methyl violet	−	−	−	−					−	−	−	−				
Safranin	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
Eosin (2,000—5,000)	+	+	+	+	+	+	+	+								
Mercurochrome	−	−	−	−	−	−	−	−	+	+	+	+	−	−	−	−
Methyl red (2,000—5,000)	+	+	+	+	+	+	+	+								
Neutral red ,,	+	+	+	+	+	+	+	+								
Indigo carmine (1,000—5,000)	+	+	+	+												
Alizarin ,,	+	+	+	+												
Nigrosin ,,	+	+	+	+												
Aniline blue ,,	+	+	+	+												
Borax carmine ,,	+	+	+	+												
Pyronin yellowish	−	−	−	−												
Fluorescein	−	−	−	−												
Methylene violet	−	−	−	−												

I = *V cholerae* (Inaba sub type)
O = *V cholerae* (Ogawa sub type)
E = El Tor vibrio
B = Basra, non agglutinating vibrio
C = *Bact coli*
T = *Bact typhosum*

experiment

1—50,000								1—100,000							
I	O	E	B	C	T	A	F	I	O	E	B	C	T	A	F
−	−	−	−	−	+	+	−	−	−	−	−	−	+	+	−
−	−	−	−	+	+	+	+	−	−	−	−	+	+	+	+
−	−	−	−	−	+	+	−	+	+	+	+	+	+	+	+
−	−	−	−	+	+	+	+	−	+	+	−	+	+	+	+
−	−	−	−	+	+	+	±	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+	+	+	+	+				
+	+	+	+	+	+	+	+								
−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
+	+	+	+	+	+	+	+								
+	+	+	+	+	+	+	+								
−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+
−	−	−	±	+	+	+	+								
−	−	−	−					−	−	−	−	+	+	+	+

A = *Bact paratyphosum A*
F = *Bact flexneri*
\+ = growth of bacteria i e no bacteriostasis
− = no growth i e positive bacteriostasis
± = Sometimes +, sometimes −

special instances the medium used was nutrient broth, serum broth or peptone-water containing glucose or saccharose, in order to see whether a dye would still be bactericidal in their presence Only complete bactericidal effects were noted Variations in results were seen when suitable dyes were not available and when the pH of the solutions was slightly altered It may be noted here that when 4 to 5 drops of culture were added to 10 c c of dye solutions in normal saline, the lethal effect, if any, was well seen, as the number of vibrios to be dealt with was exceedingly small in comparison with those present in 9 c c of culture Our standard method of assessing the real value of a dye was to add the dye to 9 c c of young actively growing culture

TABLE II

Bacteriostasis by methylene violet

Vibrio	Number of strains	1/100,000 per cent	1/500,000 per cent
Inaba sub type	18	100 positive	100
Ogawa sub type	19	85 approximately	50 approximately
El Tor	18	84 ,,	70 ,,
Para cholera	18	67 ,,	60 ,,
Saprophytic	19	43 ,,	20 ,,

Methylene violet being a sensitive dye, its colour and bacteriostatic action disappear if prepared plates are even one-day old The outstanding points in Tables I and II are —

		Inhibits
1	Brilliant green (1/100,000)	Inaba, Ogawa, El Tor and Basra vibrios and also *Bact coli* and *Bact flexneri* but not *Bact typhosum* and *Bact paratyphosum A*
	Malachite green (1/50,000)	Do
2	Crystal violet (1/100,000)	Inaba, Ogawa, El Tor and Basra vibrios but not *Bact coli*, *Bact typhosum*, *Bact paratyphosum A* and *Bact flexneri*
	Methylene violet (1/100,000)	Do
	Acriflavin (1/50,000)	Do
	Gentian violet (1/50 000)	Do
	Methyl violet (1/50,000)	Do
	Methylene blue (1/50,000)	Do
	Thionin (1/25,000)	Do
	Pyronin yellowish (1/50 000)	Do
	,, (1/100,000)	Does not inhibit any of the above
	Fluorescein (1/50,000)	Inaba, Ogawa, El Tor and a few of the non agglutinating vibrios but not *Bact coli*, *Bact typhosum*, *Bact paratyphosum A* and *Bact flexneri*
	Mercurochrome (1/5,000)	Inaba, Ogawa, El Tor, Basra vibrios and also *Bact coli*, *Bact typhosum*, *Bact paratyphosum A* and *Bact flexneri* It does not inhibit the vibrios in 1/10,000
	Safranin (1/5,000)	Inaba, Ogawa El Tor and Basra vibrios but not *Bact coli*, *Bact typhosum*, *Bact paratyphosum A* and *Bact flexneri*
	Basic fuchsin (1/5,000)	Inaba, Ogawa, El Tor, Basra vibrios and *Bact flexneri* but not *Bact coli*, *Bact typhosum* and *Bact paratyphosum A*
	Eosin Methyl red Neutral red (1/5,000)	Do not inhibit any of the above
	Indigo carmine Alizarin Nigrosin Aniline blue Borax carmine (1/1,000)	Do not inhibit Inaba, Ogawa, El Tor and non agglutinating vibrios

It will be seen from the above that red dyes have a poor bacteriostatic effect Several strains of freshly isolated Inaba and Ogawa sub-types were tested with brilliant green and acriflavin with uniform results

TABLE III

Nutrient broth culture Final dye concentration 1/100,000
Time of contact 18 *to* 20 *hours*

Dyes	I	O	C	T	F	Ae	V F
Brilliant green	—	—	+	+	+	+	+
Malachite green	—	—	+	+	+	+	+
Acriflavin	+	+	+	+	+	+	+
Crystal violet	+	+	+	+	+	+	+
Gentian violet	+	+	+	+	+	+	+

Ae = *Bact aerogenes* V F = *Vibrio fæcalis*
\+ = growth — = no growth, i e death

In experiments with the green dyes, it was often noted that if the green colour persisted and that there was some clearing, the organisms were killed On the other hand, discharge of green colour associated with increased turbidity indicated that the organisms were alive and growing

A peptone-water culture of a standard Inaba strain was treated with several dyes and the results are expressed in Table IV —

TABLE IV

Dyes	2 hours	20 hours
Brilliant green 1/100 000	D	D
Malachite green 1/100 000	D	D
Crystal violet 1/100 000	D	D
Acriflavin 1/50 000	(D)	D
Do 1/100 000	A	A
Gentian violet 1/25 000	D	D
Do 1/50 000	A	D
Thionin 1/10,000	(D)	A
Do 1/25 000	A	A
Safranin 1/25 000	(D)	A
Do 1/50,000	A	A

D = complete death (D) = almost complete death only one or two colonies are found on culture of a thick loopful
A = alive

It will be seen from Table IV that brilliant green, malachite green and crystal violet (1 in 100 000) kill Inaba sub-type of *V choleræ* in 2 hours Acriflavin (1 in 50 000) kills in 2 to 20 hours but in a dilution of 1 in 100,000 has no lethal effect

Gentian violet (1 in 50 000) kills in 20 hours but 1 in 25,000 in 2 hours Thionin (1 in 10,000) kills the majority in 2 hours but later the organisms overgrow In a dilution of 1 in 25,000 no lethal effect is seen Safranin (1 in 25 000) kills the majority in 2 hours but later the lethal effect does not persist and the organisms overgrow In a dilution of 1 in 5,000 no killing effect is seen

Having found that vibrios are very sensitive to the green dyes, the dyes were tried on a large number of strains and the results of experiments are given in Tables V, VI, VII and VIII The time of contact with the dyes was 18 to 20 hours in all the experiments

TABLE V

Culture in **peptone-water** , *dye used is* **brilliant green** *in a final concentration of* 1 *in* 100,000

Organisms	Total number of strains	Numbers dead	Numbers alive	Percentage killed
Inaba	26	20	6	77 } approximately
Ogawa	48	43	5	89 } approximately
El Tor	1	1	0	} approximately
Cholera case NAG vibrios	96	25	71	29 } approximately

NAG = non agglutinating

TABLE VI

Culture in **serum broth** ; *dye used is* **brilliant green** *in a final concentration of* 1 *in* 100,000

Organisms	Total number of strains	Numbers dead	Numbers alive	Percentage killed
Inaba	8	7	1	88 } approximately
Ogawa	11	10	1	90 } approximately
Cholera case NAG	48	5	43	10 } approximately

TABLE VII

Culture in **peptone-water** ; *dye used is* **malachite green** ; *dilution* 1 *in* 100,000

Organisms	Total number of strains	Numbers dead	Numbers alive	Percentage killed
Inaba	11	8	3	73 } approximately
Ogawa	15	12	3	80 } approximately
El Tor	1	1	0	} approximately
Cholera case NAG	35	15	20	43 } approximately
Hooghly NAG	16	0	16	0 } approximately

TABLE VIII

Culture in **nutrient broth** ; *dye used is* **malachite green** ; *dilution* 1 *in* 100,000

Organisms	Total number of strains	Numbers dead	Numbers alive	Percentage killed
Inaba	1	1	0	} approximately
Ogawa	1	1	0	} approximately
Cholera case NAG	27	6	21	22 } approximately

The above results show that most of the true cholera vibrios (Inaba and Ogawa sub-types) are highly sensitive to the green dyes About 20 to 40 per cent of para-cholera vibrios from clinical cholera cases are also highly sensitive but some of the non-agglutinating vibrios isolated from Hooghly water were tested and found all non-sensitive to the green dyes It appears therefore that the saprophytic vibrios are probably non-sensitive to the green dyes whereas those found in clinical cholera in man are mostly sensitive

Relation of dye-sensitivity to pathogenicity and antigenic structure —As stated above, most of the Inaba and Ogawa sub-types of V *cholerae* which are known to be pathogenic to man were found highly sensitive to the green dyes A few of the dye-resistant strains were tested for roughness by thermo agglutination tests and the result was negative Colony appearance suggested that they were all smooth The question of infection of the dye-resistant strains with bacteriophage was not studied

Dye sensitivity and pathogenicity to guinea-pigs and rabbits were not found uniformly correlated Some saprophytic non-agglutinating Hooghly water vibrios, although completely dye non-sensitive were found pathogenic to animals As regards invasiveness of the vibrios, dye-sensitive strains were found more commonly and early invasive into the circulation and tissues of guinea-pigs

Bactericidal action of green dyes on organisms other than the vibrios —Table IX shows the results of an experiment with brilliant green and malachite green Vibrios were also put up as controls One strain only from each species was tested

TABLE IX

Brilliant** and **malachite green** dyes 1 in 100,000 were tried separately and the results were identical Only one strain of each organism was tested Time of contact = 18 to 20 hours Culture in **nutrient broth

Organisms	Persistence of green colour	Alive or dead	Organisms	Persistence of green colour	Alive or dead
V *cholerae* (Inaba)	± ±	D	*Bact sonnei*	—	A
V *cholerae* (Ogawa)	± ±	D or A	,, *coli*	—	A
V El Tor	± —	D	,, *cloacae*	—	A
Vibrio *Basra*	± +	D	,, *aerogenes*	—	A
Case vibrio (NAG) No 114 A	+ —	D	,, *morgani*	—	A
Case vibrio (NAG) No 184 A	— ±	D	,, *asiaticum*	—	A
Case vibrio (NAG) No 182 A, 215 C & H NAG 485 A.	+ +	A	,, *carolinus*	—	A
			Ps pyocyanea	—	A
Vibrio faecalis	+	D	*Proteus vulgaris*, X19, X2 and XK.	—	A
Bact typhosum	—	A			
,, *paratyphosum* A, B and C	—	A	*Chro prodigiosum*	—	A
			Staphylo aureus, albus, citreus	—	A
Bact faecalis alcaligenes	+ ±	D or A			
,, *enteritidis*	—	A	*Streptococcus pyogenes*	—	A
,, *aberdeen*			*Strepto faecalis*	±	A
,, *stanley*	—	A	Yeast	+	D
,, *aertrycke*			*B subtilis*	—	A
,, *sendai*	—	A	*B anthracis*	+	D
Br melitensis	±	A	*Past pestis*	± +	A
Br abortus	±	A	,, *pseudotuberculosis*	—	A
Bact flexneri	—	A	,, *septica*	—	A
,, *shigae*	—	A	New castle bacillus	—	A

It is interesting to note that the dye kills anthrax bacilli and not the bacilli of the subtilis group

Selective action of green dyes on V cholerae —To 7 c c of nutrient broth, 3 c c of young broth culture of Inaba and 2 drops of culture of *Bact coli* are added and then the mixture is

incubated After 18 to 20 hours' incubation, *Bact coli* and Inaba can still be isolated on bile-salt-agar medium by direct plating But when to 6 c c of nutrient broth, 1 c c of 1/10,000 brilliant green, 3 c c of Inaba culture and 2 drops of culture of *Bact coli* are added and the mixture is incubated for the same period, the colour of the dye is sometimes discharged and only *Bact coli* can be isolated from the mixture and not Inaba

These findings show that the dye in a concentration of 1/100,000 has a selective bactericidal action on the vibrios, for in the control broth, the organisms were all alive A similar experiment was performed by taking a bigger dose of Inaba, namely 9 c c of culture in broth, and adding to it one loopful only of culture of *Bact coli* and 1 c c of 1/10,000 of brilliant green solution, the same result was obtained Malachite green behaved in the same way as brilliant green The experimental results are shown in Table X —

TABLE X

Mixture	Colour of dye	Result
7 c c nutrient broth (pH 7 0) + 3 c c of Inaba in broth culture + 2 drops culture of *Bact coli*	-	Inaba ++++, *coli* ++
6 c c nutrient broth + 3 c c culture of Inaba + 2 drops culture of *Bact coli* + 1 c c brilliant green (1/10,000)	+	Inaba —, *coli* ++
9 c c broth culture of Inaba + 1 drop culture of *Bact coli*		Inaba ++, *coli* +
9 c c broth culture of Inaba + 1 drop of culture of *Bact coli* + 1 c c brilliant green (1/10,000)	+	Inaba —, *coli* ++
culture of Inaba + 1 c c brilliant green (1/10,000)	+	Inaba —

The same result was obtained if the culture medium used was peptone-water, and malachite green was used instead of brilliant green

Importance of the colour of the dye —If the colour of brilliant green is partly or wholly discharged by adding a few drops of an alkali, the dye fails to kill vibrios even in a low dilution of 1 in 1,000 An alkali precipitates the dye and hence no dye can get absorbed by the vibrios to exert the lethal effect An experiment conducted with final concentration of the dye from 1/1,000 to 1/100,000 shows that the dye kills vibrios, but no killing effect is seen when the colour of the above concentration is discharged by an alkali either before or after the dye is added to the culture It may be mentioned here that an acid does not discharge the green colour

Effect of brilliant green on the cholera stool —Brilliant green in a dilution of 1 in 5,000 added to cholera stools (known to contain *V cholerae*) to render it slightly green is highly bactericidal to vibrios The result is given below —

No dye added to 25 samples of cholera stool	100 per cent recovery of vibrios from the samples
Dye added to the same samples	24 per cent recovery of vibrios, i e no vibrios isolated in 76 per cent of the samples

Effect of the dye on man and animals —Brilliant green (1 in 5,000) in ½-oz doses every 2 hours given to normal persons and cholera cases is usually retained and no untoward effects are observed except sometimes coloration of the urine The dye in the above concentration when administered by stomach tube in 50-c c doses every day for 3 consecutive days is non-toxic to monkeys The dye is also non-toxic to rabbits, guinea-pigs and mice

Effect of the dye in treatment of cholera cases—A solution of brilliant green (1 in 2,500) sweetened with 2 per cent cane sugar and flavoured with a little spt menth pip was given to 35 cases of cholera in ½-oz doses every hour for 6 to 8 hours A few patients did not like the mixture and a few vomited This vomiting might have been the ordinary vomiting of cholera Stools were examined before and after the dye treatment from day to day by direct plating on bile salt-agar, modified Wilson-Blair medium and by the candle-boric-peptone-water method of Panja (1942) In untreated cases vibrios generally disappeared from the 4th to the 7th day after the illness but in the dye-treated cases the organisms generally disappeared earlier in 2 to 3 days In a few of the dye-treated cases, vibrios persisted up to the 5th or 6th day No definite clinical improvement could be attributed to the dye It may be that the alkalinity of the intestinal contents inhibits the bactericidal action of the dye

Studies are being made on the action of several other dyes on vibrios

Summary

1 Brilliant green (1 in 100,000) exerts a bacteriostatic effect on Inaba and Ogawa sub-types of *V cholerae*, El Tor vibrio and the Basra strain of non-agglutinating vibrio Crystal violet and methylene violet (each 1 in 100,000) *malachite green, acriflavin, gentian violet,* methyl violet methylene blue fluorescein, pyronin yellowish (each 1 in 50 000), thionin (1 in 25,000) mercurochrome safranin and basic fuchsin (each 1 in 5 000) exert the same inhibitory effect on the above vibrios Eosin methyl red, neutral red borax carmine indigo carmine, alizarin, aniline blue and nigrosin (each 1 in 5,000) do not inhibit the vibrios Red dyes have poor bacteriostatic effect on vibrios

2 Brilliant green and malachite green dyes (1 in 100 000) exert a complete selective bactericidal effect on most Inaba and Ogawa sub-types of *V cholerae* and on large numbers of para-cholera vibrios isolated from clinical cholera cases but are harmless to non-agglutinating vibrios isolated from Hooghly water

3 The few dye-resistant strains of *V cholerae* that were tested were not found rough

4 Dye-sensitivity and pathogenicity to laboratory animals were not found uniformly correlated As a rule dye-sensitive strains were found more commonly and early invasive

5 Organisms belonging to the genera—*Bacterium*, including *salmonella* and *shigella, proteus, pyocyanea, staphylococcus streptococcus*, and *subtilis* group are not affected by the same concentrations of green dyes

6 Acriflavin, crystal violet, gentian violet (1 in 100,000) fail to exert the bactericidal effect on Inaba and Ogawa sub-types of vibrio cultures in nutrient broth

7 As a rule persistence of green colour and clearing of turbidity after contact with the organisms indicate sterility and discharge of colour and presence of turbidity signify multiplication

8 The presence of an excess of alkali in the medium prevents the bactericidal action of the green dyes

9 The green dyes (brilliant and malachite) are non-toxic to man and laboratory animals in the doses employed

10 Brilliant green added to cholera stools in a final dilution of 1/5,000, kills the vibrios in the stools Thirty-five cases of cholera were treated with the dye per mouth Vibrios in the stools disappeared earlier than in the untreated cases, but the clinical improvement was not marked as a rule, due probably to in-activation of the dye by alkaline intestinal contents

Acknowledgments

My thanks are due to Dr B C Chatterjee, the Officer-in-Charge of the Cholera Ward of the Campbell Hospital, Calcutta, for allowing me to experiment on his cases,

and to Dr B M Paul, for getting pure cultures of vibrios for me and corroborating my bactericidal results on several strains

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STUDIES IN FISH-LIVER OILS

Part I

THE BIOLOGICAL ASSAY OF VITAMINS A AND D IN GHOL- (*SCIÆNA MILES*) AND MUSHI- (*SCOLIODON SORROKOWAH*) LIVER OILS

BY

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[Received for publication, October 22, 1942]

CORRIGENDUM

Certain typographical errors have occurred in the scientific names of fishes included in Tables I to III in our earlier paper in this *Journal*, **29**, pp 279–286, 1941 The corrected words should read as follows (1) *Scoliodon sorrakowah* for *Scoliodon sorrakewah*, (2) *Harpodon nehereus* for *Harpodon neherens* and (3) *Polynemus tetradactylus* for *Polynemus tetractylus* — S P Niyogi.

methods —

TABLE I

Some constants of mushi and ghol-liver oils

Name of oil	Acid value	Saponific value	Iodine value	Unsaponifiable matter, per cent
Mushi	2 40	203	134	2 87
Ghol	1 35	184 3	136	5 00

Samples of mushi- and ghol-liver oils which gave 7,300 I U and 42,000 I U per g of oil respectively by the tintometric method were used for the biological assays of vitamins A and D

The vitamin A assay

Coward (1938) has stated that out of the several methods that are followed for the estimation of vitamin A biologically 'the increase in weight method has the distinct

advantage over the other methods in having a criterion that is easily measured' Hence the growth method was adopted in this investigation

The diet used in the estimation of vitamin A.—The diet consisted of

	Per cent
Casein (extracted with ether and alcohol)	15
Dextrinized rice starch	77
Marmite	4
Salt mixture (Osborne and Mendel, 1919)	4

The above diet was supplemented with diluted Radiostol which provided 28 I U of vitamin D per rat per week, each rat being fed twice a week—14 I U at a time The starch was mixed with sufficient water to make solid lumps and these were broken into small pieces and were baked in a pan on an ordinary gas cooking stove until brittle The starch thus dextrinized was ground and mixed in the diet The diet was served uncooked to the rats after mixing all the ingredients

Procedure —Forty-eight young rats four weeks old and weighing between 35 g and 40 g were equally divided into six groups and fed with the vitamin A-free basal diet mentioned above They were weighed once a week for the first two weeks of the preparatory period, then twice a week until the rate of growth slowed down further still and then every day till they ceased to put on weight When the weight reached a steady stage three groups out of the six were given orally a supplement of 0 223 mg , 0 446 mg and 0 892 mg of mushi-liver oil per rat per week for three weeks The animals in the other three groups, i e the control groups, received doses of standard cod-liver oil corresponding to 3 5 I U, 7 I U and 14 I U of vitamin A per rat per week for three weeks The weights of the rats in both the groups were recorded twice a week The results are given in Table II The graphs showing the increase in weight against the logarithms to the base 10 of the doses given (mg of mushi oil or I U of standard oil) were drawn on the same graph and the vitamin A content of the oil was calculated from the graph following the method adopted by Coward (*loc cit*) The same procedure was adopted in the case of ghol-liver oil The results of duplicate experiments are included in Table II —

TABLE II

The doses and increase in weight of mushi- (shark) and ghol-liver oils as compared with standard cod-liver oil and their vitamin A content

(Results are given for complete 3 weeks' test period)

	Dose of oil in mg	Increase in weight in g	Standard cod liver oil		Vitamin A content in I U per g of oil
			Dose of oil in I U	Increase in weight in g	
Mushi liver oil					
1st experiment	1 341	4 5	10 5	4 8	7,079
	2 682	8 0	21 0	9 3	
	5 364	8 9	42 0	15 6	
2nd experiment	0 670	2 4	10 5	4 5	7,440
	1 341	4 4	21 0	8 7	
	2 682	8 4	42 0	17 0	

TABLE II—*concld*

	Dose of oil in mg	Increase in weight in g	Standard cod liver oil: Dose of oil in I U	Standard cod liver oil: Increase in weight in g	Vitamin A content in I U per g of oil
Ghol liver oil					
1st experiment	0 233	3 0	10 5	3 8	32,377
	0 466	5 8	21 0	7 0	
	0 933	11 0	42 0	16 3	
2nd experiment	0 233	3 6	10 5	4 6	28,261
	0 466	7 4	21 0	9 4	
	0 933	11 3	42 0	16 1	

Note —The standard cod-liver oil used in investigation was obtained from the Nutrition Research Laboratories, Coonoor It had been spectrographically assayed and was found to contain 2,100 I U of vitamin A per g of oil

Vitamin D assay of mushi- and ghol-liver oils

The bone-ash method has been employed in the present assay of vitamin D For a discussion of the relative merits of various other methods Coward's (*loc cit*) monograph may be consulted

Since these oils contained very large quantities of vitamin A it was likely that the latter might interfere with the vitamin D assay The vitamin A was therefore destroyed by passing oxygen through the oil which was maintained at 100°C When a sample of the oil thus treated failed to give colour with $SbCl_3$ when dissolved in chloroform the bubbling of oxygen was stopped According to Drummond and Coward (1920) passing in air at 96°C for 3 hours completely destroys the vitamin

Diet —The vitamin D free basal diet had the following composition —

	Parts
Cane sugar	49
Starch	21 5
Egg-white (dried)	18
NaCl	1
$CaCO_3$	1 5
Marmite	4 0
Olive oil	4 5
Red-palm oil	0 5

This diet is principally the new rachitogenic diet of Schneider and Steenbock (1939) It has a normal calcium and a low phosphorus content Although Schneider and Steenbock published their work in 1939 the authors are not aware of attempts made anywhere to use this new diet in vitamin D assay There are two points in favour of using it in assay work (1) greater measure of success in producing rickets in rats and (2) a considerable shortening of the experimental period

Procedure —Fifty-six young rats four weeks old and weighing between 35 g and 40 g were equally divided into seven groups of eight in each and fed with the rachitogenic diet The rats in three groups received increasing doses of mushi- or ghol-liver oil as the case might be, the animals in the other three groups receiving the doses 0 175 I U , 0 350 I U and 0 7 I U of vitamin D respectively of the standard oil per rat per week for 3 weeks One group served as the negative control. The examination of the epiphyses of tibiæ of these animals showed

presence of rickets The assay period lasted for three weeks at the end of which the rats were killed, their femora removed and freed as completely as possible from the adhering tissue by washing with water and rubbing with a piece of cheese-cloth Each bone was broken into two, tied up in a piece of filter-paper and extracted in a large volume of boiling alcohol for about an hour and then with ether for at least 24 hours in the Soxhlet extractor The bones were then dried to constant weight Each pair of bones was ashed by heating in a silica crucible over a Bunsen burner to constant weight and the ash-content determined The results given by the animals in each group were averaged and the averages compared

For calculating the vitamin D content the percentage weight of ash was plotted against the logarithms to the base 10 of the doses given (mg of the unknown or I U of the standard oil) The value for vitamin D was calculated from this graph by finding out the doses for the same increase in the weight of the ash on the standard oil and the unknown The results of the assay are given in Table III .—

TABLE III

The doses and the percentage increase in weight of bone-ash for mushi- (shark) and ghol-liver oils as compared with the standard cod-liver oil in the determination of the vitamin D content of the former

	Rats on experimental oils		Rats on standard cod liver oil		I U vitamin D per g of oil
	Dose of oil in mg in three weeks	Percentage of ash	Dose of standard oil in I U in three weeks	Percentage of ash	
EXPERIMENT I					
Mushi liver oil	3 6	31 19	*Nil*	29 40	97
	7 2	33 30	0 5250	32 14	
	14 4	36 65	1 0500	35 18	
			2 1000	40 36	
EXPERIMENT II					
Ghol liver oil	0 90	32 30	*Nil*	28 98	575
	1 80	37 15	0 5250	32 22	
	3 60	35 88	1 0500	37 23	
			2 1000	40 47	
EXPERIMENT III					
Ghol liver oil	0 90	31 93	*Nil*	28 47	564
	1 80	36 52	0 5250	31 99	
	3 60	39 56	1 0500	36 88	
			2 1000	40 07	

Note —The standard for vitamin D used in these assays was a sample of the United States Pharmacopœia (U S P) reference cod-liver oil obtained directly from U S A and labelled to contain 95 U S P XI vitamin D units per g of oil The U S P XI vitamin D unit is the same as the International Unit of vitamin D

DISCUSSION

That the mushi- and ghol-liver oils were very rich in vitamin A had been shown by the tintometric assay As mentioned earlier it was necessary to prove by biological experiments

that the whole of the chromogenic substance which gave the blue colour with $SbCl_3$ was vitamin A Such experiments with the mushi-liver oil have yielded vitamin A values which are identical with those obtained by the tintometric assay In the case of the ghol-liver oil, however the biologically obtained vitamin A value was 30,320 I U per g (average of duplicate experiments), whereas the tintometric method had given a value of 42 000 I U per g (Table II) Thus, it seems that in ghol-liver oil there exists a substance which does not promote growth and which makes up nearly a third of the total chromogenic material responsible for the blue colour with $SbCl_3$

It may be of interest to discuss here some points about the interpretation of the results obtained by the various non-biological methods of assay The relationship of the Lovibond Blue unit and Sherman unit obtained biologically has been investigated by Norris and his colleagues (1929, 1932) who find no definite relationship between the two An attempt to establish a factor for interpreting the Carr-Price values in terms of I U has been made by other workers also Carr and Jewel (1933) working on a vitamin concentrate give a ratio of $\frac{\text{I U}}{\text{Blue Units}} = \frac{21}{1}$ Lathbury (1934) found a ratio of 20 to 1 for a distillate of the oil and 40 to 1 for the cod-liver oil Holmes and Corbet (1937) report a ratio of 30 to 1 on a crystalline vitamin concentrate

A similar uncertainty exists in the interpretation of the values obtained spectrographically The conversion factor of 1,600 has been accepted by the Permanent Commission of Biological Standardization of the League of Nations Seshan (1940) and Rajagopal (1941) have used this factor in converting spectrophotometric values into the International Units They compare the I U thus obtained with the Blue Units calculated by the tintometric assay The ratio of the Carr-Price value to the E (extinction coefficient) values was found to vary between 10 and 38 to 1 by Seshan , the variation was, however, less in case of oils having a large vitamin A content or when the determinations were made on the unsaponifiable matter Rajagopal found the ratio $\frac{\text{I U}}{\text{Blue Units}}$ to vary between 33 and 131 The ratio varied from sample to sample of the liver oils of sharks and saw-fish investigated It is probable, therefore, that greater discrepancies are likely to be met with if a uniform conversion factor is applied to the blue values obtained with oils from the livers of different species of fish

In view of the considerations outlined above the authors feel that whenever a new source of vitamin A is found it should be subjected to all the three methods of assay before applying any factor to convert the colorimetric or spectrophotometric values into International Units of vitamin A

So far as the vitamin D content of these two oils is concerned the assay shows that in comparison to their vitamin A content the former is almost negligible, a finding which is along the same lines as those of Ranganathan (1941)

Summary.

The liver oils from the two fish, viz mushi (*Scoliodon sorrokowah*) and ghol (*Sciæna miles*), were subjected to the tintometric and biological methods of assay for vitamin A The factor used to convert the blue values (as read) to I U was 4 2 (Bomskov, 1935) The two methods gave values which agreed in the case of mushi-liver oil The biologically obtained value for ghol-liver oil was, however, considerably lower than that given by the tintometric method

The vitamin D content of these two liver oils determined by the bone-ash method was 97 I U and 575 I U per g of oil for mushi- and ghol-liver oils respectively

Acknowledgments

The authors have great pleasure in thanking Dr W R Aykroyd, Director, Nutrition Research Laboratories, I R F A , Coonoor, for kindly supplying the reference cod-liver oil used in the vitamin A assay They are grateful to Professor George and Professor Murti for furnishing the zoological nomenclature of the fishes investigated in the present and previous inquiries

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STUDIES IN FISH-LIVER OILS

Part II

THE SEASONAL VARIATION IN THE YIELD AND VITAMIN A CONTENT OF SOME FISH-LIVER OILS

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[Received for publication, October 22, 1942]

It was noticed early during the course of the study of fish-liver oils that the vitamin A content as well as the yield of the oil from the liver of any particular variety of fish varied in different months of the year As some of these liver oils are already in use as substitutes of cod-liver oil it is essential to know to what extent variation may be expected to occur and whether the variation shows any particular trend during the year The liver oils of four varieties of fish, viz mushi (*Scoliodon sorrokowah*), ghol (*Sciæna miles*) shengti (*Macrones gluilo*) and wagli (*Dasybatus imbricatus*), were chosen for study

In every month one sample of fish liver of each variety was obtained and the extraction of the oil in duplicate and colorimetric estimation of vitamin A were carried out as described in the earlier paper (Niyogi *et al*, 1941) The figures are given in Table I —

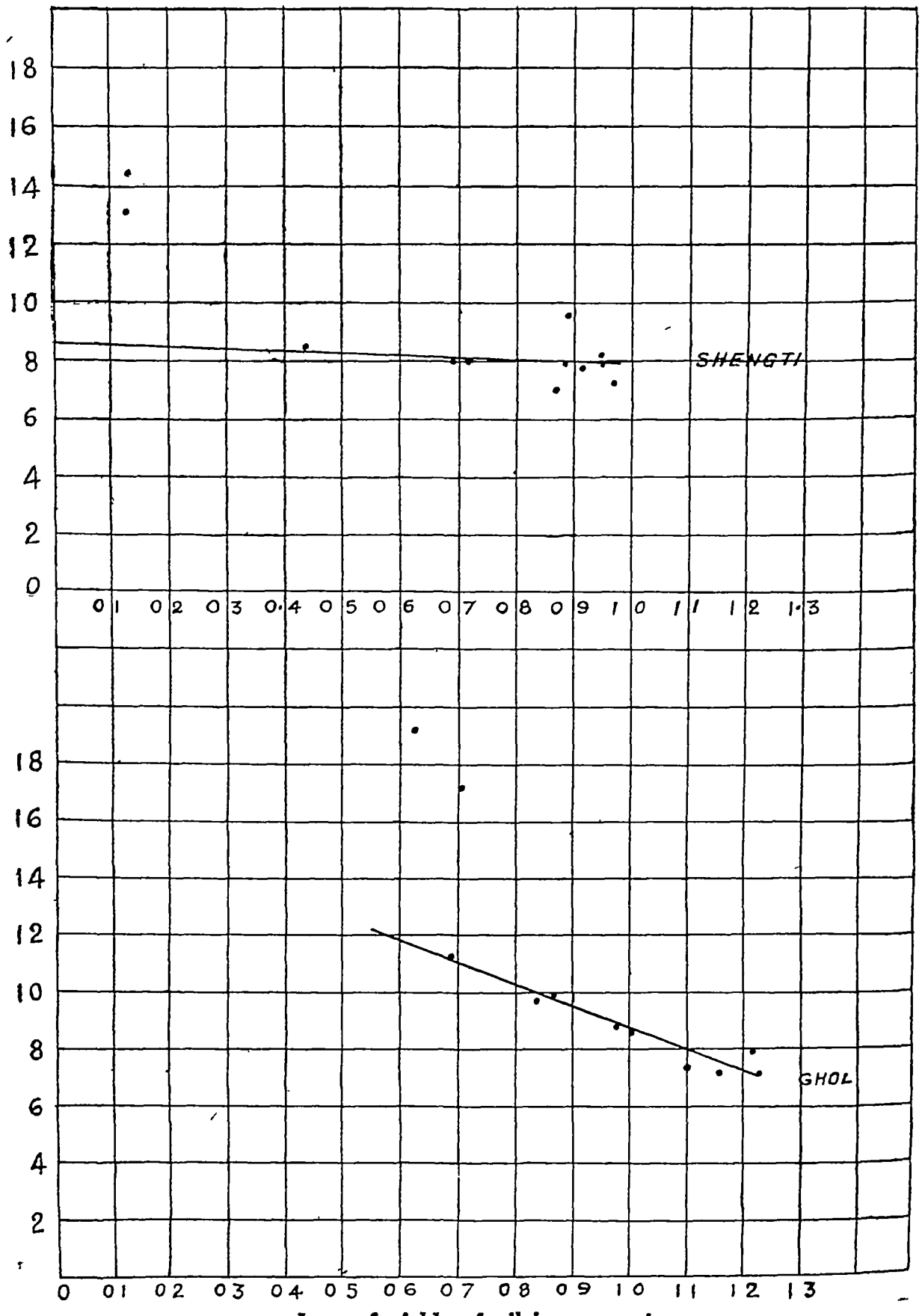
TABLE I

Monthly observations on the yield and the vitamin A content of some liver oils

Month	Mushi (*Scoliodon sorrokowah*)		Wagli (*Dasyba us imbricatus*)		Ghol (*Sciæna miles*)		Shengti (*Macrones gluilo*)	
	Oil per cent	Blue Units per g	Oil per cent	Blue Units per g	Oil per cent	Blue Units per g	Oil per cent	Blue Units per g
1940								
May	49 80	8 853	29 17	300	16 50	7 843	7 85	9,696
June	61 40	6,824	25 54	490	17 26	7,056	7 40	6,892
July	23 70	9 072	25 75	218	7 04	9,703	4 97	7,953
August	63 46	5 530	31 70	332	5 07	17 140	2 73	8,371
September	47 81	5 575	26 60	1,208	4 26	19 285	1 34	13,000
October	60 90	2 652	27 30	1,987	Sample could not be obtained		1 34	14,290
November	58 40	2,121	25 80	714	4 89	11,111	5 20	7,994
December	48 60	2,531	26 34	686	7 36	9,802	8 20	7,691
1941								
January	41 36	6 437	25 46	634	10 32	8 644	9 20	7,235
February	39 63	6 563	23 26	588	9 03	8 932	7 65	7,839
March	42 60	6 324	22 86	487	12 60	7 364	8 76	7,832
April	43 66	6,250	23 46	474	14 62	7 091	8 85	8,090

The observations extended over a period of one full year

The results obtained show that there is a variation in the yield of the oil and the vitamin A content of the latter This variation is very irregular in mushi-liver oil, it is least marked



The relationship between the yield of the oil and its vitamin A content.

in wagh-liver oil whereas with the other two fish-liver oils there is a tendency towards a decrease in the yield of oil from the livers during the monsoon This observation raises some important questions Firstly, can some reason be ascribed for these variations and, secondly, is there a relation between the yield of the oil and its vitamin A content ? In the light of the available information it is not possible to give a definite answer to either of these two questions Molteno and Rapson (1939) state that in the fish geelbeck (*Atractoscion æquidens* C and V) a decrease in total vitamin A content of the liver accompanied increased intensity of feeding Seshan (1940) observes that during the spawning season the vitamin A content of the liver oils of eight different species of fish, including sharks, is poor, while in the growth season it is fairly large There is no definite information available about the life-history and habits of any of the fish under investigation and hence no particular reason for the observation can be assigned Any of the following conditions, viz the age sex, size, feeding habit or breeding season, might probably influence the yield of oil and its vitamin A content Other workers have confined themselves to the study of changes in the vitamin A content of the oils Bills *et al* (1934) have reported that the vitamin A content of the halibut-liver oil was related to the oil content of the liver, but that the decrease in vitamin A content during certain months of the year was disproportionately greater than the increase in the oil content, suggesting that other factors in addition to dilution controlled the vitamin A concentration of the oil

In the present investigation an attempt has been made to correlate the yield of the oil with its vitamin content No relationship could be established in the case of mushi- and wagh-liver oils So far as the ghol- and shengti-liver oils are concerned, however, the vitamin A concentration was found to vary with the logarithm of the percentage yield When the two were plotted on a Graph a straight line passing through the maximum number of points was obtained The relationship may be expressed as follows —

$$\text{Vitamin A (in Blue Units per g of oil)} = K \log P + C$$

where K and C are constants and P is the percentage yield of the oil
For ghol-liver oil the expression is

$$\text{Vitamin A} = 0\,42 \log P + 9\,6$$

and for shengti, vitamin $A = 0\,04 \log P + 7\,97$

Thus, it will appear that at least in two cases the vitamin A concentration depends on the yield of oil from the livers Further work seems to be necessary to confirm this interesting observation

Summary

The seasonal variation in the yield of the oil and the vitamin A content of liver oils, of four varieties of fish, viz mushi, wagh, shengti and ghol, has been studied

The yield of the oils from the livers varied from month to month, in the case of each fish The vitamin A content also showed large variations There was no definite relation between the yield of the oil and its vitamin A content in the case of mushi- and wagh-liver oils, but in the case of shengti and ghol, the vitamin A concentration was found to increase as the yield of the oil decreased The relation between the two is given by the expression vitamin $A = K \log P + C$ where P is the per cent yield and K and C are constants which are different for the two oils

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INVESTIGATIONS ON THE FOOD VALUE OF FISH AND OTHER MARINE PRODUCTS

Part II

THE PROTEIN AND MINERAL CONTENTS

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[Received for publication, February 10, 1943]

In an earlier paper (Khorana Sarma and Giri, 1942) the values for the nicotinic acid content of various types of fish caught in the coastal waters of Waltair, which constitute an important source of food in the Northern Circars, were presented The present investigation relates to the protein and mineral contents of the muscle tissue of the fish

Experimental

Moisture—For the determination of moisture, Bidwell and Sterling's (1925) distillation method was adopted in which a modification of the tube used by Dean and Stark (1920) was employed toluene being used for the immiscible volatile liquid The results obtained by this method are very satisfactory and the duplicates agree very well (*see* Allen's 'Commercial organic analysis', 1932)

Nitrogen was determined by the Kjeldahl method

Calcium was estimated by the usual method of precipitating the calcium as calcium oxalate and titrating with potassium permanganate

Phosphorus—The total phosphorus was determined by the method of King (1932) by digesting the tissue with perchloric acid and estimating the inorganic phosphorus colorimetrically by the method of Fiske and Subbarow (1925) after neutralization

Iron was determined by the method of Kennedy (1927)

Copper was estimated by the method described by Tompsett (1934, 1935), which is based on the di-ethyl-di-thiocarbamate reaction of Callan and Henderson (1929) as modified by McFarlane (1932)

The results obtained are summarized in the Table The values given are the average obtained on two estimations

Discussion

The protein and mineral contents of fish available in other parts of the country have been investigated Saha and Guha (1939) have investigated the moisture, fat, mineral matter protein, ionizable iron, total iron, calcium and phosphorus of 24 different varieties of Bengal fresh-water fish In a subsequent paper these authors (Saha and Guha, 1940) have presented the results of their studies on the food value of 13 different kinds of Bengal fish Niyogi *et al* (1941) and Appanna and Devadatta (1942) have carried out the analysis of fresh fish from Bombay coastal waters Mitra and Mittra (1941) have reported the results of their analysis of some fish consumed in Bihar

TABLE

Food values of the muscle tissue of fish

Number	Common name	Local name	Zoological name	Weight of each fish (average), g	Weight of muscle tissue, g	Moisture, per cent	Protein, per cent	Ash, per cent	Calcium, per cent	Phosphorus, per cent	Iron mg, per cent	Copper mg, per cent
1	Seer	Vanjaram	*Scomberomorus*			74 9	22 37	1 63	0 006	0 157		
2	Shark	Sorra				72 8	21 85	1 50	0 007	0 267		0 067
3	Jew fish	Gorasalu	*Sciænidæ*	25	5 8	80 2	18 10	1 45	0 077	0 254	0 994	0 050
4	Horse mackerel	Para	*Caranx*	117	67	76 0	21 0	1 60	0 021	0 235		
5	Ribbon fish	Savallu	*Trichiurus haumela*	230	55	79 3	19 1	1 72	0 032	0 275		0 230
6	Cat fish	Jellalu	*Siluridæ*	310	175	77 1	21 44	1 33	0 010	0 225		0 246
7		Golavindalu				78 1	20 3	1 41	0 021	0 150		0 143
8	Pomfrets	Chanduva	*Stromateus*	200	64	78 5	19 1	1 55	0 020	0 291	0 90	traces
9	Silver bellies	Karalu	*Leiognathus*	364	92	77 4	22 5	1 73	0 019	0 339	0 72	
10	Mullets	Bonthalu	*Mugil* Spp	110	43	76 0	22 6	1 29	0 025	0 296	1 15	0 08
11	Pollona	Engallu	*Pellona* Spp	345	95	75 7	21 8	1 41	0 043	0 276	0 60	0 012
12	Sardines	Kavallu	*Sardinella fimbriata*	7 6	3	78 1	21 0	1 65	0 090	0 356	2 52	traces

It will be seen from the Table that the values obtained for the calcium content are considerably lower than those given by Saha and Guha (1940) for Bengal fish and are of the same order as those obtained for fish analysed in Coonoor Laboratories (Health Bulletin No 23) and those given by Niyogi *et al* (*loc cit*) and Appanna and Devadatta (*loc cit*) for Bombay fish It remains to be seen if the age or the locality influences the content of calcium The figures obtained for protein phosphorus iron and copper are however, in agreement with those values given by other workers in India The copper content of ribbon fish and cat fish is considerably higher than that of other types of fish investigated

Summary

The protein and mineral contents of a number of economically important food fishes in the Northern Circars have been determined The protein content ranges from 19 to 23 per cent, Ca, 0 006 to 0 090 per cent, phosphorus, 0 150 to 0 350 per cent, iron, 0 6 mg to 2 5 mg per cent and copper 0 01 mg to 0 24 mg per cent The values indicate that these fish constitute a good source of protein phosphorus and iron

Acknowledgments

The authors are grateful to the Inspector of Fisheries, Vizagapatam, for kindly arranging the supply of fish for these investigations

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AVAILABILITY OF CALCIUM IN LADY'S FINGER (*HIBISCUS ESCULENTUS*), CABBAGE (*BRASSICA OLERACEA CAPITATA*) DRUMSTICK (*MORINGA OLEIFERA*) AND AMARANTH TENDER (*AMARANTHUS GANGETICUS*)

Part I

AVAILABILITY OF CALCIUM IN VEGETABLES DETERMINED BY EXPERIMENTS ON GROWING RATS

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[Received for publication, January 27, 1943]

INTRODUCTION

In average or typical diets a deficiency of calcium is more likely to occur than a deficiency of any other element The usual diet of average people in India and other eastern countries consists mainly, if not entirely, of cereals supplemented with pulses and vegetables Cereals and pulses, although rich in phosphorus, are poor sources of calcium In fact metabolism experiments performed (Basu, Basak and De, 1941 , Basu, Basak and Rai Sircar, 1939) in this Laboratory showed that typical rice diets providing on an average 234 mg of calcium and typical wheat diets providing about 300 mg of the element, per adult daily, failed to maintain an adult in calcium equilibrium The addition of about 10 oz cow s milk to the daily diet brought the experimental subjects into calcium balance It must be mentioned, however, that even this small amount of milk is beyond the means of the average Indian

A known adequate source of calcium would be the vegetables which supplement the cereals and pulses in the dietary The problem of finding some vegetables rich in calcium which would be well utilized in the system is a very urgent one The present investigations deal with the availability of calcium of some vegetables rich in this element which grow in abundance in India

A number of papers have appeared on the utilization of vegetable calcium during the last two decades References to earlier investigations will be found in the papers by Sherman and Hawley (1922), Fincke and Sherman (1935), Kao, Conner and Sherman (1938) and by Fairbanks and Mitchell (1938) A perusal of these papers shows that the evidence regarding the availability of vegetable calcium is conflicting Whether the calcium of a particular vegetable can be well utilized or not by animals and human beings can be settled only by direct experiments

The present investigations were carried out to determine the availability of calcium in four commonly used vegetables, viz lady's finger (*Hibiscus esculentus*)—Beng Dhenras, cabbage (*Brassica Oleracea capitata*)—Beng Bandha kopi, drumstick (*Moringa Oleifera*)—Beng Sajney, and amaranth tender (*Amaranthus gangeticus*)—Beng Lal shak or Dhulla shak Of these amaranth has a very high calcium content (about 0 3 per cent on moist basis) The other vegetables are fairly good sources of calcium No work has yet appeared in India on the availability of calcium from these or other vegetables The object of this investigation was to find whether milk could be fully or partly replaced by any of these vegetables so far as the supply of calcium was concerned

The availability of the calcium of these vegetables was investigated by two different methods In the first place young healthy albino rats which had been reared on the usual diet of the laboratory were placed on five different diets in which milk and the four vegetables were respectively the only sources of calcium In the case of the drumstick diet only, milk supplied 50 per cent of the total calcium At 8 weeks of age the rats were killed and the utilization of calcium was determined and compared This method throws light on the relative value of the vegetables in promoting calcification in young growing rats and probably, therefore, in growing children

A separate investigation was undertaken to find out how the calcium of these vegetables was utilized in maintaining calcium balance in human beings The metabolism experiments were performed on an adult The experimental subject was first given a basal diet containing inadequate amounts of calcium The effect of supplementing the basal diet with each of the different vegetables was then observed

Availability of calcium in vegetables determined by experiments on growing rats

The vegetables were purchased from the local market, thoroughly washed first in tap-water and then in distilled water and dried in an oven at a temperature of about 70°C to avoid charring The dried vegetables were finely ground, the whole lot thoroughly mixed and finally stored in stoppered glass-jars in a refrigerator In the case of amaranth, the leaves and tender stems were separately dried and ground and the two lots were thoroughly mixed In the case of cabbage two lots were prepared, one containing more of the outer green leaves and the other more of the inner greenish leaves After drying, the two lots were ground and thoroughly mixed In the case of lady's finger and drumstick the entire vegetable was used for the experiment Milk was used in the form of skimmed milk powder and the same lot was used throughout the investigation Butter-fat in the form of ghee purchased from the local market was separately added

The powdered vegetables, the skimmed milk powder and also the Ca-free casein used in preparing the diets were analysed The results are shown in Table I As the experiment could not be conducted on rats with amaranth, the rats refusing to take the diet, no data appear for the dry powder of this vegetable in the Table

Table I

Analysis of skimmed milk, vegetables and casein

Composition	Skimmed milk, per cent	Lady's finger, per cent	Cabbage, per cent	Drumstick, per cent	Casein, per cent
Calcium	1 510	1 155	0 750	0 395	
Phosphorus	0 963	0 583	0 398	0 451	0 85
Protein (N × 6 25)	44 40	16 97	16 10	19 45	
Oxalic acid		0 050	0 035	0 042	
Moisture content of fresh vegetables		85 540	92 980	75 340	

In fresh condition lady's finger contained 0 112 per cent Ca and 0 0072 per cent oxalic acid, amaranth 0 328 per cent Ca and 0 10 per cent oxalic acid, cabbage 0 059 per cent Ca and 0 0025 per cent oxalic acid and drumstick 0 097 per cent Ca and 0 0103 per cent oxalic acid

Methods

In all cases calcium was determined by the McCrudden (1911-12) method by precipitating calcium as oxalate at pH 4 8 to 5 2 using methyl-red as indicator and titrating the oxalic acid with potassium-permanganate solution Phosphorus was determined by titration with uranium acetate according to Pincus (1859) and Malot (1887) and nitrogen by the usual Kjeldahl method Oxalic acid was determined by precipitation with calcium chloride in the presence of acetic acid and subsequent titration of the calcium oxalate with potassium-permanganate solution as described by Mazumdar and De (1938)

Healthy young rats (albino), 4-weeks old, which had been reared on the stock laboratory diet consisting of whole wheat, whole cow's milk and vegetables with a bi-weekly addition of cod-liver oil and Marmite were placed in individual iron cages with raised bottom and maintained on the experimental diets for 4 weeks

Table II gives the composition of the experimental diets and the salt mixture The diets were so planned that they contained almost the same percentage of calcium, phosphorus and protein The calcium phosphorus ratio was always 1 1 The calcium of the diets was derived entirely from milk or from the vegetables under investigation The calcium content of drumstick powder was, however, so low that in this case 50 per cent of the calcium was derived from skimmed milk and 50 per cent from drumstick as in the experiments of Fincke and Sherman (*loc cit*) on the availability of vegetable calcium Reliable results regarding availability of calcium from a particular source are obtained by employing a diet the calcium content of which is derived entirely from the foodstuff under investigation and in this respect the diets employed in this investigation are to be preferred to those employed by Sherman The fibre contents of the diets were not adjusted since it was found by several workers that fibre was without any influence on calcium retention The diets were preserved in stoppered glass-jars and stored in a r frigerator

TABLE II

Composition of the diets

Composition	Diet I per cent	Diet II per cent	Diet III per cent	Diet IV per cent
Casein	12 4	17 60	16 50	2 40
Butter fat	10 0	10 00	10 00	10 00
Starch	50 1	37 87	23 15	28 31
Salt mixture	5 0	5 00	5 00	5 00
Skimmed milk powder	22 5			11 25
Lady's finger powder		29 44		
Cabbage powder			45 35	
Drumstick powder				43 04
TOTALS	100 0	100 0	100 0	100 0
Calcium	0 34	0 34	0 34	0 34
Phosphorus	0 32	0 32	0 32	0 32
Protein (N × 6 25)	22 39	22 7	23 8	20 77

Vitamins A, D and B complex were supplied twice a week

Diet I—milk, diet II—lady's finger, diet III—cabbage diet IV—drumstick.

Composition of the salt mixture

	Parts
Sodium chloride	4 40
Magnesium sulphate	5 45
Potassium chloride	7 80
Ferric citrate	1 18

The animals were chosen so that the average initial weights of the group of rats to be compared were approximately the same In all cases littermates of the same sex were compared Food and distilled water were supplied to the rats *ad libitum* and records were kept for the food supplied Any food spilled was carefully separated from the fæces and collected In each case the total amount of food spilled was taken into consideration while calculating the total food intake Records were kept of the growth of the animals during the experiments and are shown in Table III It was observed that, while all the animals were apparently in good health, the average growth rates of rats on the milk diet were slightly higher

TABLE III

Average growth of rats from age 4 weeks to 8 weeks on different diets

Diet	Sex	Number of rats giving average	Average initial body weight, g	Average final (net) body weight, g	Average net gain in weight, g	Average total food intake, g	Average gain per g food, g
I	M	8	38 0±1*	111±7*	72 ±4*	200	0 36
	F	7	35 0±1	91±5	56 ±3	194	0 28
II	M	6	43 0±3	97±4	54 ±1	208	0 26
	F	6	34 0±0 5	81±1	46 ±0 5	204	0 22
III	M	6	37 0±1	94±4	58 ±3	194	0 30
	F	5	34 0±2	91±2	57 5±2	206	0 28
IV	M	7	38 0±1	84±2	46 ±2	196	0 23
	F	5	33 5±0 01	78±2	45 ±2	200	0 22

* Average deviation of the mean

Diet I—milk, diet II—lady's finger, diet III—cabbage, diet IV—drumstick

At 8 weeks of age the rats were weighed, killed with chloroform and brushed to remove any adhering food particles from the fur The gastro-intestinal tract was dissected and the contents removed The weight of the contents subtracted from the final live weight gave the net weight which was used as a basis in calculating the percentage of body calcium of 8-week old rats The alimentary tract was discarded as the calcium in the walls of the intestinal tract was negligible The animals were ashed in silica basin first over a low flame and finally in an electric muffle furnace at dull red-heat The ashes were dissolved in (1 4) hydrochloric acid, filtered and diluted to 500 c c in volumetric flasks Aliquots of the solution were analysed for calcium by the method already stated

The results are summarized in Table IV The bodies of male rats on the milk diet (diet I), lady's finger diet (diet II), cabbage diet (diet III) and drumstick diet (diet IV) contained respectively 0 75 ± 0 002, 0 79 ± 0 006, 0 81 ± 0 002 and 0 83 ± 0 002 per cent of calcium The values for females were respectively 0 83 ± 0 004, 0 86 ± 0 003, 0 84 ± 0 002 and 0 86 ± 0 003 It will be noticed that rats on the milk diet had the lowest percentage of body calcium and those on the drumstick diet the highest This is to be expected since the rats on the milk diet grew at the most rapid rate and those on the drumstick diet most slowly as is evident from Table III The intermediate values for the percentage of body calcium of rats on lady's finger and cabbage diet was also in conformity with their growth rates

TABLE IV

Calcium content of 8 week old rats fed on diets containing the same amount of calcium from milk or vegetables

Diet	Sex	Number of rats giving average	Average net body weight, g	Average Ca intake, g	Average total body calcium, g	Average per cent body calcium
I	M	8	111 ± 7*	0 676 ± 0 037*	0 826 ± 0 046*	0 75 ± 0 002*
	F	7	91 ± 5	0 653 ± 0 034	0 766 ± 0 048	0 83 ± 0 004
II	M	6	97 ± 4	0 706 ± 0 013	0 769 ± 0 029	0 79 ± 0 006
	F	6	81 ± 1	0 693 ± 0 004	0 689 ± 0 007	0 86 ± 0 003
III	M	6	94 ± 4	0 658 ± 0·024	0 751 ± 0 034	0 81 ± 0 002
	F	5	91 ± 2	0 693 ± 0 019	0 770 ± 0 025	0 84 ± 0 002
IV	M	7	84 ± 2	0 670 ± 0 022	0 664 ± 0 031	0 83 ± 0 002
	F	6	78 ± 2	0 681 ± 0 024	0 673 ± 0·016	0 86 ± 0·003

* Average deviation of the mean

Diet I—milk, diet II—lady's finger, diet III—cabbage, diet IV—drumstick.

To obtain the storage of calcium it was necessary to know the initial calcium content of the body of the animals just before the beginning of the experiment This was obtained by determining the percentage of body calcium at 28 days of age of littermates of the animals reared to 8 weeks on the experimental diets The method adopted to determine the total body calcium of these animals were exactly the same as described previously for the experimental animals The results are given in Table V It will be observed that the percentage body calcium of 4-week old rats of the same litter was practically unaffected by the sex difference But the value slightly differed from litter to litter This fact was taken into account while calculating the initial body calcium of the experimental animals The fact that sex difference was without any effect on the percentage of body calcium of 4-week old rats, was in close agreement with the results obtained by Kao, Conner and Sherman (*loc cit*) who obtained 0 74 ± 0 008 per cent of body calcium for male rats at 28 days and 0 74 ± 0 004 per cent for female rats of the same age The values obtained in the same laboratory by Sherman and McLeod (1925) and by Sherman and Booher (1931) on comparable animals were 0 67 per cent for males and 0 74 per cent for females The values obtained by us give an average value of 0 60 ± 0 003 per cent for both sexes This value is somewhat low in comparison with the values obtained in Sherman's laboratory

TABLE V

Calcium content of 4-week old rats of different litters on the stock laboratory diet

Litter number	Sex	Body weight, g	Total body calcium, g	Per cent body calcium
1	M	45·0	0 2854	0 63
2	M	37 5	0 2254	0 60
3	M	35 0	0 1995	0 57
4	M	38 5	0 2333	0 61
6	M	38 5	0 2322	0 60
7	M	37 5	0 2220	0 59
8	M	38 0	0 2290	0 60
9	M	37 5	0 2252	0 60
2	F	34 5	0 2099	0 61
5	F	42 5	0 2720	0 64
8	F	33 5	0·2023	0·60

For an exact basis of comparison a 'calcium utilization factor' was calculated for each of the diets This was obtained by dividing the calcium retention by the intake The average calcium utilization factors for each of the diets are given in Table VI The values for males were 0 87 ± 0 005 for the milk diet, 0 82 ± 0 004 for the cabbage diet, 0 71 ± 0 003 for the lady's finger diet, and 0 70 ± 0 002 for the drumstick diet The values for females were respectively 0 84 ± 0 008, 0 81 ± 0 004, 0 70 ± 0 004 and 0 69 ± 0 002 With milk diets having the same percentage of calcium Fincke and Sherman (*loc cit*) obtained the utilization factors of 0 86 for males and 0 77 for females and Kao, Conner and Sherman (*loc cit*) obtained the value 0 88 for both males and females

TABLE VI

Utilization factor of calcium in milk and the various vegetables

Diet	Sex	Number of rats giving average	Average utilization factor for calcium
I	M	8	0 87 ± 0 005*
	F	7	0 84 ± 0 008
II	M	6	0 71 ± 0 003
	F	6	0 70 ± 0 004
III	M	6	0 82 ± 0 004
	F	5	0 81 ± 0 004
IV	M	7	0 70 ± 0 002
	F	6	0 69 ± 0 002

* Average deviation of the mean

Diet I—milk, diet II—lady's finger, diet III—cabbage, diet IV—drumstick

From the results in Table VI it is quite evident that sex difference did not appreciably influence the utilization of calcium of milk and in the case of the vegetables the influence was almost *nil* The difference of the utilization factor of calcium of lady's finger from that of milk is 0 16 for males and 0 14 for females For drumstick these values are 0 17 for males and 0 15 for females These differences are quite significant It therefore appears that the calcium of lady's finger and drumstick are not quite as well utilized as that of milk Nevertheless, the two vegetables serve as a fairly good and available source of calcium, the availability of the calcium in these two vegetables being nearly four-fifths of that of the calcium in milk

In the case of cabbage the differences of the calcium utilization factors from those of milk are 0 05 for males and 0 03 for females These figures are not so significant and it appears that the calcium of cabbage is probably almost as well utilized as that of milk The low oxalic acid content of these vegetables is probably associated with the food utilization of the calcium which they contain

Tender amaranth contains a remarkably high amount of calcium (about 0 3 per cent on moist basis) But, in spite of the best efforts, investigations with this particular vegetable were impossible since, most surprisingly in the case of so omnivorous a species, the rats refused to take the diet containing this vegetable The small amount of food taken by a few did not produce any increase in weight, the rats became very pale and feeble, and actually in some cases a decrease in weight was observed The utilization of calcium from this vegetable was studied on the human subject and the results are incorporated in Part II of this paper (Basu and Ghosh, 1943)

SUMMARY

Healthy young rats, 4-week old were placed on four diets in one of which all the calcium was supplied entirely by skimmed milk In the case of two other diets the milk was entirely replaced by enough finely ground dried cabbage or lady s finger to provide the same amount of calcium and in the fourth, half of the skimmed milk was replaced by enough ground dried drumstick to provide the same amount of calcium as in the milk diet At 8 weeks of age the animals were killed and their bodies analysed for calcium

Comparison of the availability of calcium in these vegetables with that of milk was made by calculating for each an utilization factor which is the ratio of calcium retention to intake The values for males were 0 87 for milk diet, 0 71 for lady's finger, 0 82 for cabbage and 0 70 for drumstick diet The values for females were respectively 0 84, 0 70, 0 81 and 0 69 Sex difference was, therefore, practically without any appreciable effect on the utilization The calcium of cabbage was almost as well utilized as that of milk The calcium from the other two vegetables, namely lady s finger and drumstick, was also fairly available The rats refused to take the amaranth diet

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AVAILABILITY OF CALCIUM IN LADY'S FINGER (*HIBISCUS ESCULENTUS*), CABBAGE (*BRASSICA OLERACEA CAPITATA*), DRUMSTICK (*MORINGA OLEIFERA*) AND AMARANTH TENDER (*AMARANTHUS GANGETICUS*)

Part II.

AVAILABILITY OF CALCIUM IN VEGETABLES DETERMINED BY METABOLISM EXPERIMENTS ON A HUMAN ADULT

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THE conclusions arrived at from the experiments with young rats described in Part I of this paper (Basu and Ghosh, 1943) will very probably apply to young growing children Nevertheless, metabolism experiments on human beings deriving the major portion of their calcium from the vegetable under investigation will directly indicate the value of the vegetable in maintaining calcium balance in adults The results obtained with adults are very likely to be generally applicable to children as well

EXPERIMENTAL

The experiments were conducted on a healthy young man (G C N, 20 years) weighing 49 kilo In each of the experiments, to avoid any effect of the previous diet, the subject was given a basal diet of adequate energy content but deficient in calcium for a preliminary period of three days in which no collection of urine and fæces was made During the subsequent three-day periods the subject consumed the basal diet, then the basal diet supplemented by one of the vegetables—*Amaranthus gangeticus*, *Hibiscus esculentus* (lady's finger), *Brassica Oleracea capitata* (cabbage) and *Moringa Oleifera* (drumstick)—or milk for one or two periods as the case was The basal diets used represented typical Indian diets one (1) a rice-fish diet containing 600 mg rice, 60 mg dhal, 200 mg ordinary vegetables, 70 mg fish and 30 mg mustard oil and the other (2) a vegetarian diet containing 600 mg rice, 70 mg dhal, 215 mg ordinary vegetables and 30 mg ghee (butter-fat) The rice consumed by the subject throughout each experiment was supplied from the same stock The vegetables were secured fresh each day and aliquots were dried and powdered for analysis The amount of calcium in the water used for cooking and drinking was also taken into consideration The phosphorus content of water was negligible Weighed amounts of food were cooked every day and consumed by the subject *in toto* in two portions In the case of drumstick the refuse from mastication was carefully collected, dried and analysed On the average, it was found that 6 mg of calcium were lost in that manner This was subtracted from the total intake through this vegetable In the case of cabbage, the green outer leaves and the inner greenish leaves were thoroughly mixed in almost equal proportions before cooking

The subject was in good health and exhibited little change in weight during the experimental period

Technique and analytical methods were similar to those used in previous investigations from this Laboratory (Basu, Basak and Rai Sircar, 1939)

Discussion of results

The metabolism data are summarized in the Table Each period included three consecutive days and only the mean of the daily analytical figures for each period is presented The chronological order of the experimental periods is denoted by arithmetical numbers after the letter P The average retention in per cent of calcium from a vegetable or milk supplement is indicated in the last column The data in the Table indicate that the calcium of the vegetables, lady's finger, cabbage and drumstick, is probably more or less equally utilized to maintain calcium equilibrium in adults although the percentage utilization is much lower than that of milk calcium Amaranth, in spite of its high oxalate content, appears to be a fairly good and available source of calcium

Table

Metabolism data showing the utilization of vegetable and milk calcium by G C N

The figures represent average daily intake and output in mg

Diet	Period	Intake			Calcium output			Calcium balance	Average retention of calcium from the supplement in per cent
		Ca	P	Ca/P value	Urine	Faeces	Total		
Basal rice fish diet	P_1	320	1237	1/3 8	10	228	238	+ 82	
Basal diet + 200 g amaranth	P_2	976	1359	1/1 4	14 6	650 3	664 9	+311 1	
Basal diet + 200 g amaranth	P_3	976	1359	1/1 4	15	540 0	555 0	+421	
	Average P_2 and P_3	976	1359	1/1 4	14 8	595 3	610 1	+366 9	43 2
Basal vegetarian diet	P_1	203	1105	1/5 4	29 3	248 4	277 7	− 74 7	
Basal diet + 200 g lady's finger	P_2	442	1267	1/2 9	32	399 6	431 6	+ 10 4	
Basal diet + 200 g lady's finger	P_3	442	1267	1/2 9	32 3	432	464 6	− 22 6	
	Average P_2 and P_3	442	1267	1/2 9	32 1	415 8	447 9	− 5 9	28 8
Basal vegetarian diet	P_1	163 7	1065	1/6 5	18 6	150 9	169 5	− 5 8	
Basal diet + 200 g cabbage	P_2	341	1149	1/3 4	26	247 7	273 7	+ 67 3	
Basal diet + 200 g cabbage	P_3	341	1149	1/3 4	36	302	338	+ 3 0	
	Average P_2 and P_3	341	1149	1/3 4	31	274 9	305 9	+ 35 1	23 1
Basal vegetarian diet	P_1	196 5	1105	1/5 6	29	234	263	− 66 5	
Basal diet + 200 g drumstick	P_2	385 3	1330	1/3 5	39	364 3	403 3	− 18 0	
Basal diet + 200 g drumstick	P_3	385 3	1330	1/3 5	44	368	412	− 26 7	
	Average P_2 and P_3	385 3	1330	1/3 5	41 5	366 2	407 7	− 22 4	23 4
Basal vegetarian diet	P_1	199 5	1105	1/5 5	28 3	269 1	297 4	− 97 9	
Basal diet + 10 oz milk	P_2	564 5	1415	1/2 5	47	496	543	+ 21 5	
Basal rice-fish diet	P_1	310	1237	1/4	7	231	238	+ 72	50 1
Basal diet + 10 oz milk	P_2	675	1547	1/2 3	16	341	357	+ 318	

This investigation shows that with the vegetarian rice-pulse diet a supplement of about 200 g to 300 g (about ¾ seer) of either cabbage or lady's finger or drumstick brings an adult from negative to positive calcium balance Amaranth is a very rich source of calcium and a supplement of about 100 g of this vegetable would suffice The same purpose would also be served by about 10 oz (5 chhataks) of milk The non-vegetarian diet containing rice and fish contains about 120 mg more calcium than the vegetarian diet and therefore requires supplementing with less amounts of vegetable or milk

Summary

Calcium metabolism experiments were conducted on a healthy adult to find whether the calcium in lady's finger, cabbage, drumstick and amaranth (leaves and tender stems) could be utilized to maintain the calcium equilibrium in human adults These vegetables were given as supplement to two types of basal diets representing the typical Indian diets—one containing rice and fish and the other purely vegetarian All the vegetables had a favourable effect on calcium balance and brought the Ca P ratio to more favourable values Amaranth, in spite of its high oxalate content, served as a fairly good available source of calcium Comparison with milk showed that except in the case of amaranth the utilization of calcium in the vegetables was much lower than that of the calcium in milk

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THE DETERMINATION BY CHEMICAL METHODS OF THE FOOD VALUES OF YET ANOTHER BATCH OF EDIBLES

BY

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(From the Nutrition Scheme, Public Health Laboratories, Patna)

[Received for publication, September 28, 1942]

INTRODUCTION

In spite of a good deal of work which has been carried out in different laboratories in India the estimation of the food values of all the local edibles has by no means been completed Since the last of the series of communications on the subject published from this Laboratory (Mitra and Mittra, 1942) giving the food value of 80 edibles, no other paper on the subject has been published in India

EXPERIMENTAL

In the present investigation 50 different edibles consisting of 2 kinds of grain foods, 14 of flesh foods, 9 of fruits, 10 of leafy vegetables, 9 of root vegetables and 6 of other vegetables have been analysed by chemical methods on the lines detailed in a previous communication (Mitra, 1938) from this Laboratory The results are shown in the appended Table

DISCUSSION

Of the three kinds of prawns and lobsters analysed, *karuana gorla* (*prawn*) and *crab* seem to be very rich in calcium In this connection it may be noted that the samples of karuana gorla analysed contained a certain amount of the chitinous jacket, and in the case of crab every effort was made to remove completely the calcareous shell though small bits may have remained In the case of both these flesh foods the dressing of the meat was done to the extent practised in Indian homes The meat of the crocodile was found to be consumed by the low-class riverine population The calcium content of the different leafy vegetables was found to vary between 531 mg /100 g in the case of *paruar sag* and 119 mg /100 g in the case of *alu sag* The tubers analysed grow wild in the table land of Chotanagpur and were mostly found to be consumed by poorer class of people whenever the family stock of grains fell short 'Other vegetables', with the solitary exception of *mougri*, were not found to be popular with the people of the upper social classes

ACKNOWLEDGMENT

The authors are obliged to Dr Baini Prasad, O B E, Director, Zoological Survey of India, Calcutta, and Dr S K Mukherjee, Curator of the Herbarium, Royal Botanic Garden, Calcutta, for kindly furnishing the correct Latin names, and to Rai Bahadur Dr B P Mozoomdar, Director of Public Health, Bihar, for his interest in the work and encouragement

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TABLE

Food values of edible portions in grammes per cent

Serial number	Hindi name or local name of the food stuff	English name	Latin name	Moisture, g	Protein, g	Ether extractive, g	Carbohydrates, g	Mineral matter, g	Calcium, g	Phosphorus, g	Crude fibre, g
	GRAIN FOODS										
1	Bhet	Seeds of water lily	*Nymphea lotus*	9 99	8 25	0 98	75 73	0 87	0 022	0 112	4 18
2	Math		*Phaseolus aconitifolius* Jacquin	6 77	24 60	0 72	61 71	3 40	0 152	0 210	2 80
	FLESH FOODS										
3	Abhua kachhua	Turtle's meat	*Lissemys punctata* Bonnaterre	76 96	18 86	1 19	1 78	1 21	0 043	0 196	
4	Andail gorla	Prawn	*Palæmon* sp	75 46	21 51	1 72		1 31	0 038	0 249	
5	Chaha (bara)	The ruff and reeve	*Philomachus pugnax* Linn	70 34	25 94	2 26		1 46	0 003	0 321	
6	Dhonr kachhua	Turtle's meat	*Kachuga tectum* Gray	80 21	18 11	0 72		0 96	0 006	0 142	
7	Gharial ka gost	Meat of narrow snouted crocodile	*Gavialis gangeticus* Gmelin	77 43	20 57	0 40	0 33	1 31	0 013	0 162	
8	Gorha, harwa or katwa	Turtle's meat	*Hardella thurgi* Gray	81 20	14 68	2 83	0 33	0 96	0 004	0 150	
9	Haran ka gost	Venison	*Antilope cervicapra* Linn	75 33	21 00	0 58	1 90	1 19	0 003	0 233	
10	Karuana gorla	Prawn	*Palæmon lamarrei*	76 57	17 51	2 06	1 15	2 71	0 741	0 258	
11	Kekra	Crab	*Paratelphusa (Barytelphusa) jacquemontii*	61 74	11 93	17 30	4 57	2 46	0 598	0 675	
12	Katah kachhua	Turtle's meat	*Trionyx gangeticus* Cuvier	81 43	15 24	0 32	1 87	1 14	0 010	0 175	
13	Rehu machhli ka anda	Roes of rohu fish	*Labeo rohita*	64 29	26 36	5 95	2 18	1 22	0 004	0 223	
14	Sakchi fish		*Dasyatis* sp	80 57	16 72	0 52	1 20	0 99	0 005	0 155	
15	Sal kachhua	Turtle's meat	*Kachuga kachuga* Gray	77 12	15 41	2 29	4 19	0 99	0 007	0 149	
16	Tengul gorla	Lobster	*Palæmon* sp	77 28	20 47	0 87		1 38	0 016	0 279	
	FRUITS										
17	Harfarowrie		*Phyllanthus distichus* Muel Arg	91 20	0 73	0 61	5 89	0 52	0 006	0 006	1 05
18	Hoormed		*Erycibe paniculata* Roxb	15 97	2 78	1 09	76 95	0 88	0 145	0 080	2 33
19	Kamalgatha	Lotus seeds (green and mature)	*Nelumbium speciosum*	84 58	3 85	0 68	8 92	1 12	0 049	0 151	0 85
20	Neem		*Melia azadirachta*	81 88	1 26	0 98	15 22	0 66	0 025	0 041	
21	Nisorha		*Cordia myxa* Linn	82 51	1 76	0 99	12 21	2 21	0 040	0 060	0 32

22	Papa		*Gardenia latifolia*	46 85	3 74	3 09	35 00	1 86	0 021	0 091	9 16
23	Piyal		*Buchanania latifolia*	74 25	2 15	0 79	19 62	1 71	0 078	0 028	1 45
24	Sapatu	Sapodilla plum	*Achras sapota*	71 68	1 03	0 71	23 75	0 62	0 009	0 005	2 21
25	Toont	Mulberry	*Morus indica*	84 70	2 12	0 24	11 02	1 00	0 066	0 047	0 62
	VEGETABLES (LEAFY TYPE)										
26	Alu sag	Leaves of potato	*Solanum tuberosum*	88 00	1 13	0 86	3 58	1 81	0 119	0 053	1 32
27	Chimti sag		*Polygonum plebium* R Br	83 22	3 23	0 67	6 90	3 88	0 194	0 048	2 10
28	Koila karha sag		*Asteracantha longifolia* Nes	87 19	3 04	0 36	5 26	2 79	0 330	0 021	1 36
29	Konar sag		*Bauhinia purpurea*	78 11	3 60	0 95	9 78	2 05	0 312	0 092	5 51
30	Konhra ka phool	Flowers of pumpkin	*Cucurbita maxima*	89 13	2 22	0 81	5 72	1 39	0 120	0 057	0 73
31	Nisorha ka phool		*Cordia myxa* Linn	79 56	1 70	0 15	9 13	2 55	0 171	0 116	3 31
32	Parwar sag		*Trichosanthes dioica*	80 45	5 40	1 07	5 84	3 02	0 531	0 073	1 22
33	Patua sag		*Corchorus capsularia* Linn	81 43	5 11	1 13	7 97	2 72	0 241	0 093	1 64
34	Sarli sag (lupu)	Dried sarli sag	*Vangueria spinosa* Roxb	7 46	7 65	6 11	57 71	7 67	1 388	0 082	13 10
35	Sowa sag		*Peucedanum sowa*	85 15	3 36	0 67	6 83	2 47	0 218	0 074	1 52
	VEGETABLES (ROOTS AND TUBERS)										
36	Edel	Tapioca	*Manihot utilissima*	69 84	1 38	0 26	26 10	0 98	0 045	0 033	1 44
37	Epedong sanga		*Peucedanum nagpurensis* Prain	91 56	2 17	0 21	1 78	1 60	0 277	0 059	2 68
38	Goa mundakam		*Dioscorea alata* var *rubella*	73 34	1 03	0 09	23 96	0 80	0 006	0 022	0 78
39	Jipoo sanga		*Habenaria cammelinifolia* Wall	94 13	0 55	0 10	4 67	0 19	0 011	0 007	0 36
40	Kapu sanga		*Dioscorea sativa* L	67 77	3 34	0 17	26 43	1 27	0 014	0 094	1 02
41	Murum sanga		*Dioscorea spinosa* Roxb	82 54	1 36	0 09	14 65	0 64	0 024	0 024	0 72
42	Ochen sanga		*Momordica cochinchinensis*	88 63	1 49	0 10	7 56	1 10	0 064	0 089	1 12
43	Salukad pundi	Water lily (white)	*Nymphea lotus* var *lotus*	62 45	3 11	0 30	31 71	1 30	0 065	0 217	1 13
44	Salukad jenga	Water lily (red)	*Nymphea lotus* var *rubra*	49 13	4 12	0 33	43 33	1 55	0 076	0 220	1 54
	VEGETABLES (OTHER VARIETIES)										
45	Bagnaha		*Capparis horrida* Linn	66 96	6 05	3 78	11 71	1 99	0 064	0 081	9 57
46	Hatnah sirni	Tetralobar bean	*Lotus tetragonolobus* L	90 58	1 86	0 39	4 87	0 50	0 032	0 021	1 80
47	Kachha boorooi		*Gardenia gummifera*	81 13	1 47	0 19	14 06	0 81	0 030	0 018	2 34
48	Kapu		*Dioscorea sativa* I	69 26	2 23	0 12	26 08	0 99	0 018	0 083	0 72
49	Kochra, koin or dola	Fruits of mahua	*Bassia (Madhuca) latifolia*	74 42	2 15	1 15	19 66	1 11	0 051	0 022	1 51
50	Mougri	Rat tailed radish	*Raphanus sativus* var *caudatus*	92 33	1 28	0 25	4 30	0 72	0 078	0 024	1 12

DETERMINATION OF THE BIOLOGICAL VALUE OF PROTEINS FROM RED ANTS (*ŒCOPHYLLA SMARAGDINA*) BY THE BALANCE-SHEET METHOD

BY

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[Received for publication, September 28, 1942]

INTRODUCTION

SOME time ago it was suggested to the senior author (K M) by one of the eminent nutrition workers in India that it would be interesting to find out how far the proteins from young red ants (*Œcophylla smaragdina*), which are consumed by certain groups of population in Bihar (Mitra and Mittra, 1942), were available to the human organism A visitor to any of the *hatias* (village markets) in the district of Singhbhum can see for himself that these ants with their eggs sell like hot cakes though the price is comparatively dear *Hau* (as these ants are known) is often eaten raw and is considered to be a delicacy, particularly by the *Ho* and local Oriya children

EXPERIMENTAL

Hau was purchased from the *hatias* in Kolhan area (Singhbhum district) and was thoroughly dried in the sun after all the extraneous matter had been carefully picked out The dried material was then ground into powder of uniform consistency, stocked in a stoppered glass-phial and preserved in the refrigerator for use On analysis by chemical methods 100 g of the dried powder was found to contain moisture, 4 05 g , nitrogen, 8 74 g , ether extractives, 15 23 g , mineral matter, 4 42 g , and carbohydrate (by difference), 21 65 g

TABLE

The average intake and output of nitrogen per rat per day in g

Rat unit number	NITROGEN FREE DIET			EXPERIMENTAL OR TEST DIET				Relative digestibility coefficient	Relative biological value
	Dry food intake, g	NITROGEN EXCRETED g		Dry food intake, g	Total nitrogen intake, g	NITROGEN EXCRETED, g			
		Fæces	Urine			Fæces	Urine		
				10 *per cent level*					
1	12 5	0 0315	0 0366	9 8	0 1724	0 094	0 0836	63 8	57 2
2	11 7	0 0340	0·0348	12 2	0 2298	0 1148	0 1067	64 8	51 8
3	12 2	0 0323	0 0255	10 4	0 1862	0 0826	0·0660	73 0	70 2
4	13 4	0·0288	0 0389	9 5	0 1913	0 0881	0 0896	69·0	61·6
9	8 9	0 0273	0 0316	9 5	0 1666	0 0973	0 0797	58 0	50 2
10	9 2	0·0278	0 0357	9 3	0 1605	0·0903	0 0762	61 1	58 7

TABLE—*concld*

Rat unit number	NITROGEN FREE DIET			EXPERIMENTAL OR TEST DIET				Relative digestibility coefficient	Relative biological value
	Dry food intake, g	NITROGEN EXCRETED, G		Dry food intake, g	Total nitrogen intake, g	NITROGEN EXCRETED, G			
		Fæces	Urine			Fæces	Urine		
				15 per cent level					
5	10 7	0 0323	0 0348	9 6	0 2490	0 1175	0 1051	65 8	57 1
6	9 9	0 0267	0 0318	11 2	0 2968	0 1331	0 1295	64 2	48 7
7	5 8	0 0179	0 0449	10 2	0 2645	0 1202	0 1061	61 3	62 3
8	9 5	0 0287	0 0357	8 3	0 2138	0 0946	0 1000	69 2	56 5
11	8 1	0 0235	0 0348	8 7	0 2232	0 1057	0 1034	63 2	51 3
12	8 3	0 0221	0 0258	10 4	0 2713	0 1151	0 1009	65 7	57 9

The biological value at 10 and 15 per cent levels of intake was determined on laboratory-bred adult white rats according to the methods detailed in a previous communication (Mitra and Mittra, 1942*a*) *The nitrogen-free diet* could not be made absolutely nitrogen free as the starch used was found to contain 0 05 per cent of nitrogen, and 1 2 mg nitrogen daily was consumed by each rat through Marmite solution, a rich source of the vitamin B complex The test diets at 10 and 15 per cent levels consisted of —

	10 per cent g	15 per cent g	
Powdered hau	111 5	167 3	(Approximate
Starch	257 0	245 0	calorific value
Sugar cubes	54 0	54 0	2,500)
Coco-nut oil	77 1	55 2	
Calcium carbonate	6 0	6 0	
McCollum's salt mixture	24 0	24 0	

In addition to the above each animal on the nitrogen-free and test diets received three drops of cod-liver oil and 2 c c of a 1 per cent solution of Marmite daily to guard against known vitamin deficiencies

RESULTS

No appreciable difference could be found between the digestibility coefficient figures at 10 and 15 per cent levels of intake The difference noticed in biological value is more

	10 per cent level	15 per cent level
Mean relative digestibility coefficient	65 0	64 9
Mean biological value	58 3	55 6

apparent than real On analysing the data statistically ' t ' was found to be equal to 0 7444 and according to Fisher's table (Fisher, 1934) (with 10 degrees of freedom) P was found to lie between 0 5 and 0 4 , consequently the *difference could not be proved to be significant* It may thus be safely presumed that the increase in biological value noticed at the 10 per cent level (as compared to that of 15 per cent) could have occurred by chance alone

SUMMARY

The digestibility coefficients and biological values of proteins from red ants (*Œcophylla smaragdina*) have been studied at 10 and 15 per cent levels of intake No appreciable difference could be found in the average figures obtained at the two levels of protein intake

ACKNOWLEDGMENTS

The authors are obliged to Rai Bahadur Dr B P Mozoomdar, Director of Public Health, Bihar, for his interest in the work and encouragement, and to Mr S Chatterjee of Bacteriophage Laboratory Patna, for kindly looking after the animals

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NUTRITION AND ITS BEARING ON PREVENTABLE BLINDNESS AND EYE DISEASES IN BENGAL

BY

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[Received for publication August 5, 1942.]

THIS clinical investigation has been carried out to determine the rôle of vitamin A deficiency in relation to preventable blindness and eye diseases in Bengal.

Disorders of the eye due to defective nutrition have been known for a long time but the nature of the deficiency has been only recently recognized. The treatment of nightblindness by ox liver is recorded in an Egyptian papyrus (Eber's) of about 1500 B.C. Hippocrates recommended 'raw ox liver dipped in honey once or twice or as much as is able.' Ancient Hindus recommended sheep's or goat's liver. In China and Japan extracts of hen's liver in honey and livers of sheep and hens were much used in the old days.

Livingstone (1857) (quoted by McCollum and Simmonds, 1925) described an affection of the eyes of some men of his party carrying out exploration in Africa whose diet consisted for a long time of sugarless coffee, manioc roots, gluten and starch. The following are quoted by Blegvad (1924). Baizeau (1861), a French Army Surgeon, gave cod-liver oil to soldiers suffering from epidemic hæmeralopia with good results. Bitot (1863) described 29 cases of xerosis conjunctiva, with hæmeralopia in older children, in a foundling hospital in Bordeaux. Gama Lobo (1865) described diseases of the eyes amongst poorly nourished slaves on coffee plantations in Brazil. Blessig (1866) observed many cases of xerosis conjunctiva and keratomalacia amongst Russian workers during the lenten fast and was of the opinion that derangements of nutrition were the cause. Thalberg (1883) observed keratomalacia in nurselings whose mothers lived under very bad nutritional conditions on account of the Russian lenten fast. Baer (1901) cured a case of keratomalacia in a child nourished on oatmeal gruel with cow's milk and lime water. Hamburger (1902) cured a similar case with nurse's milk. Mori (1904) (quoted by McCollum and Simmonds, 1925) observed 1,400 cases of xerophthalmia in Japanese children between 2 and 5 years of age during a period of partial famine. They developed xerosis and keratomalacia, a syndrome known as 'hikan' in Japan. It occurred amongst people whose diet was largely of vegetable origin and 'hikan' was rarely observed amongst fishermen on the coast, where good fish was available. Administration of cod-liver oil gave prompt relief. Chicken's liver and eel fat were also found to be effective as remedies. Mori attributed the ocular lesions to inadequacy of fat in the diet. Schiale (1907) (quoted by Blegvad, *loc. cit.*) observed that suckling children could be cured without any treatment if the mothers were given cod-liver oil.

Bloch (1919) described 50 cases of severe malnutrition with xerophthalmia in children in the vicinity of Copenhagen. Their diets consisted of separated skimmed milk, practically free from fat, pasteurized and cooked again at home, oatmeal gruel and barley soup. Bloch concluded that these conditions were due to lack of fat in the diet, since the eye conditions could be relieved by the administration of cod-liver oil, whole milk or cream mixtures. The same worker performed a human experiment corresponding to feeding experiments in animals.

He divided 32 children of ages between 1 and 4 into 2 batches The diets were the same except at breakfast That of batch 1 contained no milk or any other animal or vegetable fat To batch 2, some whole milk and some vegetable fat were supplied Within 2 months, 8 cases of xerosis developed in batch 1, which was cured by adding cod-liver oil to the diet Zak (1917), Hift (1918) and Mesiner (1919) (all quoted in the Report of the Medical Research Council, 1932) and all Austrian doctors and prisoners of war in Russia during the last war found nightblindness very common amongst Russian peasants during the religious fast before Easter, when all animal food including milk and butter is forbidden The diet was strictly vegetarian but included fats and oils of vegetable origin They found lightly cooked liver and cod-liver oil were very efficacious in the cure of nightblindness

McCarrison (1920) stated that xerophthalmia occurs not infrequently in India on a diet of rice and vegetable oil and is curable by cod-liver oil Wright (1922) described conjunctival pigmentation as one of the most characteristic early signs in vitamin A deficiency Blegvad (*loc cit*) recorded a number of cases of xerophthalmia traceable to deficiency of vitamin A and curable by administration of cod-liver oil He pointed out that the quantity of vitamin A in mother's milk varied with the quantity of the vitamin in her food If the mother's food was devoid of or deficient in fat-soluble vitamin A, then her own reserve supply would gradually become exhausted and both she and her baby would be liable to develop keratomalacia Parallel results were obtained by Drummond (1918) and Nelson, Lamb and Heller (1922) in experiments with laboratory animals

Pillat (1929) observed 70 cases of nightblindness occurring among 209 Chinese soldiers out of 3,000 examined in a military camp north of Peiping The nightblindness was associated in all of these cases with pigmentation, xerosis, wrinkling of the bulbar conjunctiva and Bitot's spots, either alone or in combination Their diets, though sufficient in quantity, were lacking in vitamin A He obtained good results in these cases by giving green vegetables and cod-liver oil He considered nightblindness, pigmentation of the fornix and the semi-lunar fold of the conjunctiva, Bitot's spots, wrinkling of the bulbar conjunctiva and pre-xerosis of the cornea to be due to vitamin A deficiency More recently, extensive clinical observations on conditions associated with avitaminosis A have been recorded by many workers, especially by Wright in India, Pillat in China, Bloch and Widmark in Denmark and Mori in Japan The following clinical conditions have been described in cases of vitamin A deficiency —

Nightblindness, in the lids, blepharitis, styes, comedones, and meibomitis, in the conjunctiva, pigmentation, xerosis, Bitot's spots, loss of lustre and wrinkling of the bulbar conjunctiva, in the cornea, xerosis, diminished sensitivity of the corneal epithelium, keratomalacia, dystrophy of the cornea, affections of the para-ocular glands especially the lacrimal and the accessory lacrimal glands

These clinical changes have also been reproduced experimentally in animals fed on vitamin A deficient diets by numerous workers The chief ocular signs and symptoms of vitamin A deficiency are discussed individually in the sections which follow —

Nightblindness —The essential feature is a defect in dark adaptation and to measure it several kinds of biophotometers and adaptometers have been devised All cases of defective dark adaptation and its advanced stage, nightblindness, are not due to vitamin A deficiency Diseases of the eye, such as opacities in the cornea, lens and vitreous, diseases affecting the peripheral parts of the retina, and choroid or both, retinal detachment, optic neuritis, optic atrophy, glaucoma and advanced myopia cause nightblindness It may also occur as a congenital condition, in Oguchi's disease, and in psychological conditions in association with neurasthenia and other functional disorders (Wittkower *et al*, 1941) Nightblindness due to vitamin A deficiency should really be called nutritional nightblindness It has been known to occur in an acute form in people living on a vitamin A deficient diet and exposed for prolonged periods to the glare of the sun either direct or reflected from the sea Elliot (1920) wrote, 'It is accepted as an axiom that nightblindness is worse in proportion to the exposure to bright light that a patient has suffered during the day' He attributes it to the activity of the visual rays of the sun Aykroyd (1930) observed this condition amongst

fishermen in Newfoundland and Labrador, whose diets were obviously deficient in vitamin A and recorded that fishermen exposed for long periods to the glare of the sun in open boats were the worse sufferers He stated that these people were aware that nightblindness could be cured rapidly by eating the fresh livers of sea gulls or of fish

The causation of nutritional nightblindness is of interest The effect of strong light in precipitating the condition and its rapid appearance when the appropriate treatment is given suggest that the visual purple is concerned Visual purple is bleached by light and is regenerated in darkness The rapidity of regeneration of the visual purple determines the onset of the vision in darkness (scotopic vision) so that nightblindness may be due to delayed regeneration of the visual purple or to its *functional deficiency* Delay in regeneration of visual purple has been demonstrated experimentally by Fridericia and Holm (1923) and Tansley (1933) in animals fed on a diet deficient in vitamin A Other workers have demonstrated either a high vitamin A or carotene content in hog's retina (Yudkin, Kriss and Smith, 1931), carotenoid pigment in ox retina (von Euler and Hellstrom 1933) vitamin A in the retina and the pigment layers of the retina of sheep, ox and pig (Wald 1933) carotene in ox retina and the pigment epithelium layers (von Euler and Adler 1934) vitamin A and xanthophyl in large quantities in the light adapted frog's retina Wald and others seem to think that vitamin A deficiency leads to functional deficiency and that delayed regeneration of visual purple is the result Other workers (Huges, Lienhardt and Aubel, 1929, Mellanby 1934) have described degeneration in the optic nerve-bundle in advanced avitaminosis A in animals and suggested that this may be the cause of nightblindness but the fact that dramatic cure occurs after treatment with cod-liver oil for a few days seems to be inconsistent with this view

Pigmentation of the conjunctiva —Several observers in the East have described a peculiar pigmentation of the bulbar conjunctiva and fornix as an early sign of vitamin A deficiency Wright (*loc cit*) described pigmentation of the conjunctiva as one of the most characteristic early signs Pillat (1929) observed a combination of nightblindness with a peculiar brown pigmentation of the conjunctiva the semi-lunar fold and the fornix He observed that 'pigmentation is also present in the beginning stages of avitaminosis and forms, if present, a very important diagnostic symptom', he also observed 'pigmentation as the only symptom of avitaminosis' Other observers holding the same view are Mori (1924) and El-Togby and Wilson (1933) This type of pigmentation has not been described in Western countries The pigment is melanin and appears in the basal cell-layers and later all layers become diffusely pigmented

Dhurandhar and Boman-Behram (1940) stated that 72 per cent of their cases with conjunctival pigmentation were deficient in vitamin A Kirwan, Sen and Biswas (1941) are of opinion that pigmentation of the conjunctiva with or without Bitot's spots is not necessarily a sign of vitamin A deficiency

Xerosis conjunctiva (xerophthalmia, conjunctivitis arida of Mackenzie) —This is a degenerative condition characterized by dryness of the conjunctiva due to impairment of the secretory activity of the mucous membrane, and not due to diminished lacrimal secretion The dryness affects the epithelium only and so the condition is known as xerosis epithelasis and is distinct from xerosis parenchymatosa The xerosis is associated with debility or malnutrition Although the nature of the malnutrition is indefinite, it has been considered a deficiency state because of its frequent association with nightblindness and because it responds to treatment either with liver or cod-liver oil On the discovery of *Bacillus xerosis* in 1874, the condition was attributed to infection with this organism This view was short-lived as *Bacillus xerosis*, though present in large numbers in xerosis of the conjunctiva, was found to be saprophytic It was again revived after Findlay (1926) observed a definite deficiency in lysozyme in the tears of vitamin A deficient rabbits In more recent years workers have produced xerosis and all stages of keratomalacia in animals fed on a diet deficient in fat-soluble vitamin A (Harden and Zilva, 1918, Osbourne and Mendel, 1913, 1914, 1915, 1921, Yudkin and Lambert, 1923, Woolbach and Howe, 1925, Green and Mellanby, 1928, 1930, and others) Their observations have supported the view of McCollum and Simmonds (1917) who held that xerophthalmia should be regarded as a deficiency disease Mori (1922) observed shrunken lacrimal and

Harderian glands in his experimental animals and revived the view that the xerosis was a result of desiccation due to the loss of tears, but clinically many cases of xerosis and keratomalacia have been observed with lacrimation Moreover, removal of the lacrimal glands does not lead to xerosis Mellanby (1930, 1933) considered the conditions to be neurotrophic In experimental animals with well-developed xerophthalmia, he found changes in the myelin sheaths of the trigeminal nerves In early cases, the nerves return to normal on a diet with vitamin A or carotene, in more severe cases typical Wallerian degeneration occurs along with degenerative changes in the cells of the Gasserian ganglion

The first detailed clinical description of the condition was given by Bitot (*loc cit*) Four stages are described (1) loss of lustre appears as a dullness of the conjunctival reflex, accentuated by keeping the lids open and most marked on the temporal side of the limbus, (2) wrinkling of the conjunctiva, which is observed in the palpebral fissure and appears concentric to the limbus, (3) Bitot's spots, which are small white sharply defined patches, triangular in shape with the base towards the limbus They are on the temporal side and bilateral They are covered with a material resembling dried foam which is made up of Meibomian secretion and can be easily removed by rubbing They, however, quickly return after removal and are not moistened by the tears, (4) leathery appearance of the conjunctiva which is thrown into folds concentric with the limbus The pathological process is one of metaplasia and hyperplasia and ultimately keratinization of the conjunctival epithelium

Keratomalacia —This has been described by a large number of previous observers as an advanced stage of xerophthalmia Pillat (1929) described (1) a pre-xerotic stage in the cornea, the signs of which are loss of lustre, dryness on exposure to the air and reduced sensitivity of the entire cornea, (2) a stage of xerosis, the signs of which are dryness of the corneal epithelium with a patchy distribution of grey areas especially marked in the palpebral areas, (3) and lastly, keratomalacia proper, the signs of which are dull cornea, formation of infiltrations, ex-foliation of the corneal epithelium and ulcer formation, frequently resulting in atrophy of the eye

Clinical investigation

The observations in this investigation were made firstly on ocular manifestations and secondly on other associated conditions The ocular manifestations are classified as follows —

GROUP I —Hæmeralopia without any pathological change in the eye or in the optic nerve

GROUP II —The conjunctival manifestations Loss of lustre, xerosis, Bitot's spots and pigmentation either alone or in combination

GROUP III —The corneal manifestations, described by Pillat as 'pre-xerosis', which consist of loss of lustre, dryness on exposure to the air and diminished sensitivity of the corneal epithelium, with or without any other manifestation in the conjunctiva

GROUP IV —Keratomalacia without breaking down of the corneal epithelium, associated with dryness of the corneal epithelium and areas of degeneration in the deeper layers of the cornea—the pre-ulcerative stage

GROUP V —Keratomalacia with breaking down of the cornea This is called the stage of ulceration

GROUP VI —Complete loss of the eye resulting in an anterior staphyloma of the eyeball or phthisis bulbi in the absence of infection, or panophthalmitis when infection has occurred

The number of cases in each group —A total of 1,604 cases were investigated and the data relating to 965 cases will be considered These include in group I 200 cases, in group II

398 cases in groups III to VI 227 cases The remaining 140 cases who showed no evidence of vitamin A deficiency, served as controls for cases examined for dark adaptation Each group will be discussed separately, in relationship to sex, age community and other associated conditions

Limitation of institutional statistics —From institutional statistics, it is difficult to establish the incidence of a disease amongst different groups of people The community and the sex composition of the population can be ascertained from the Census figures As the communal and the sex prejudice in attending clinics cannot be estimated, it is necessary to make some sort of assumption regarding these biases and for this reason the incidence of a disease deduced from hospital statistics can at best be taken only as a very rough approximation of its incidence in the general population

Group I —*Nightblindness*

Two hundred cases were observed In 51 cases, there were pathological conditions in the eyes These consisted of 36 cases of disease of the retina and 15 cases of disease of the optic nerve, making 25 5 per cent, which is a high proportion One hundred and ten cases had normal fundi, in the remaining 39 cases, the fundi were not examined Dark-adaptation tests with the biophotometer were carried out in 106 cases Table I gives the result —

Table I

Result of dark-adaptation tests in nightblindness

	Normal	Border line	Deficient	Not done	Total
Fundus healthy	14	9	69	18	110
Fundus not examined	1	6	7	25	39

Eleven out of 18 cases of the group with normal fundi in which biophotometric examinations were not done, were below 10 years of age It will be seen that in 78 cases of the group with normal fundi the existence of nightblindness was definitely established and in 13 cases of the other group, in which condition of the fundi was unknown It is questionable if all these cases were cases of nutritional nightblindness They are referred to as doubtful cases The importance of the biophotometer as an instrument for investigating vitamin A deficiency is evident from the above findings Biophotometers are now being used by even non-medical men in India to find out the degree of nutritional deficiency in the population In 51 cases, i e 25 5 per cent, the nightblindness was due to pathological conditions in the fundus Of the 106 cases examined by the biophotometer, 15 gave normal readings The occupations of the 15 cases of nightblindness with normal biophotometric readings were as follows 8 schoolboys between the ages of 9 and 21, 2 schoolgirls of 9 and 13 years, 1 tailor (18 years), 1 jute-mill worker (22 years), 1 carpenter (25 years), 1 excise vendor (42 years), 1 married woman (23 years)

Although they came to the hospital complaining of difficulty of seeing at night, the existence of nightblindness could not be confirmed by the biophotometer They were given either cod-liver oil or shark-liver oil and did not again return to hospital for treatment It is possible that during the biophotometric examination they stated that they saw the spots before they actually did so On the other hand, 56 out of the control group of 140, i e 40 per cent, showed impairment of dark adaptation on the biophotometric examination The control group had no signs or symptoms of vitamin A deficiency It is therefore clear that the diagnosis of nightblindness by biophotometer readings has its limitations There may be faulty interpretation of biophotometer readings in cases in which there is disease of the eye and the optic nerve

Sex incidence in nightblindness cases —Table II shows the sex incidence in 78 definite and 13 doubtful cases of nightblindness —

TABLE II

Sex incidence in nightblindness cases

Sex	Definite cases	Doubtful cases	TOTAL
Male	74	13	87
Female	4	*Nil*	4
TOTAL	78	13	91

According to the 1931 Census figures the number of males in Calcutta is twice that of females The preponderance of males has therefore to be attributed either to (1) variation in the sex incidence of the disease or (2) prejudice on the part of females against attending hospitals It seems, therefore, that the incidence of nightblindness in males is higher, as prejudice on the part of the female sex against hospitals would not explain the ratio of 1 11 This appears to corroborate the effect of exposure to glare in the development of nightblindness

Age group —The age distribution of 78 definite and 13 doubtful cases were as follows (Table III) —

TABLE III

Age grouping in nightblindness cases

	Less than 10 years	Less than 15 years	Less than 20 years	Less than 25 years	Less than 30 years	Less than 35 years	Less than 40 years	Less than 45 years	Over 45 years	Not known	TOTAL
Definite cases	6	12	16	19	12	6	1	2	2	2	78
Doubtful cases	2	1	3	1	1	3	1	*Nil*	1	*Nil*	13
TOTAL	8	13	19	20	13	9	2	2	3	2	91

Biophotometric examination was not carried out as a rule in children below the age of 10 years The percentage of cases of nightblindness in different age groups amongst males above 10 years of age with their relative age distribution in the population of Calcutta is given in Table IV —

TABLE IV

Percentage in age group and comparison with the percentage in the population of Calcutta

Age	Less than 15 years	Less than 20 years	Less than 25 years	Less than 30 years	Less than 35 years	Less than 40 years	Less than 45 years	Over 45 years	TOTAL
Number of cases	13	19	19	11	9	2	3	2	78
Percentage	16 7	24 4	24 4	14 1	11 5	2 6	3 7	2 6	100
Percentage in population of Calcutta	8 4	12 6	15 9	16 3	11 4	10 3	8 2	13 9	100

The proportion of cases in age groups below 25 years of age is higher than in the population and it is just the reverse for age groups over 25 years It appears that males below the ages of 25 years suffer more than those above that age

Community —The community distribution of definite and doubtful cases of nightblindness is given in Table V —

TABLE V

Community distribution in nightblindness cases

	Hindu	Mohammedan	Christian	Others	TOTAL
Definite cases	59	10	8	1	78
Doubtful cases	10	3	Nil	Nil	13
TOTAL	69	13	8	1	91

The Hindu population of Calcutta is 2 6 times more than the Mohammedan The number of Hindu cases is 5 4 times greater than that of Mohammedan cases As Mohammedans attend hospital in smaller numbers than Hindus the incidence of nightblindness in the two communities is probably about the same

Treatment —In all cases of vitamin A deficiency, a definite plan of treatment was followed and the following medicines were used In all slight cases without any gastro-intestinal or liver disorders cod-liver oil or shark-liver oil was administered The shark-liver oil was secured from Madras and its potency for vitamin A was found to be four times and for vitamin D twice that of cod-liver oil Its chemical composition apart from vitamin content was found to be almost the same as that of cod-liver oil The adult dose was 2 drachms of cod-liver oil 3 times a day or ½ drachm of shark-liver oil 3 times a day All severe cases and all cases with gastro-intestinal or liver disorders were treated by parenteral administration of vitamin A The preparations used were 'Prepalin' made by the Glaxo Laboratories, each c c containing 100,000 International Units of vitamin A and later in the course of the inquiry a vitamin concentrate prepared by Dr Aykroyd in the Nutritional Research Laboratories in Coonoor Out of 78 definite cases of nightblindness, 20 cases could not be treated Fifty-eight cases were treated as follows (Table VI)

TABLE VI.

Treatment of nightblindness cases

Treatment	IMPROVEMENT		DURATION OF TREATMENT				
	No	Yes	Less than 1 week.	Less than 2 weeks	Less than 1 month	Less than 3 months	Failed to attend
Cod liver oil, 20 cases		12			6	6	8
Shark liver oil, 28 cases		16			10	6	12
'Prepalin', 7 cases	1	6	5	1			
Vitamin A concentrate, 3 cases		3	1	1	1		

Re-examination by the biophotometer was carried out in 27 cases All of these gave normal readings Eight out of the 13 cases belonging to the doubtful group were treated Three cases were cured and 5 did not re-appear

GROUP II —*Conjunctival manifestations*

Pigmentation of the conjunctiva —The colour varies from a light-brown to a mahogany brown and it has been observed that when a patchy light-brown colour is found on the bulbar conjunctiva in the palpebral fissure, then the colour in the lower fornix and caruncle is much darker Later, dark-brown pigmentation appears around the limbus The colour in the lower fornix, caruncle and around the limbus may be so dark as to resemble argyrosis and it is often mistaken for it In these extreme cases the pigmentation of the bulbar conjunctiva is uniform ly dark-brown and the mucous membrane is dull in appearance The pigmentation does not affect the palpebral conjunctiva and is due to melanin This pigmentation has not been observed in Western countries The strong glare of the sun in the tropics may be responsible for the production of pigment in the potential melanoblasts in the basal layers of the conjunctiva, especially at the limbus and caruncle (Pillat, 1933)

Xerosis of the conjunctiva —The first sign of this is lack of lustre of the bulbar conjunctiva most marked on the temporal side of the palpebral fissure This spreads in patches, wrinkling appears, the conjunctiva is thrown into folds in the direction of the movement of the eyeball and finally Bitot's spots appear They are usually light-grey but sometimes white in colour, are always bilateral and may vary in colour and extent in both eyes They appear as spots and as these increase they form an oval patch which later may develop into a triangular one with the base towards the limbus They are always to be seen on the temporal side The patch is covered with a substance which resembles in appearance dry white foam This can be rubbed off showing the Bitot's spots It, however, quickly re-forms and is mainly composed of Meibomian secretion

Three hundred and ninety-eight cases belonging to this group were examined for dark adaptation by the biophotometer and the results are given in Table VII —

TABLE VII

Results of the dark-adaptation test in cases showing conjunctival manifestations

Condition	Normal	Border line	Deficient	TOTAL
Pigmentation alone	112	40	41	193
Xerosis alone	4	*Nil*	1	5
Pigmentation and xerosis	73	25	25	123
Xerosis and Bitot's spots	*Nil*	1	1	2
Pigmentation, xerosis and Bitot's spots	47	17	11	75
TOTAL	236	83	79	398

Assuming that all border-line cases in this group are deficient in vitamin A, it has been found that 236 out of 398, i e 59 3 per cent, were normal as regards dark adaptation In the control group of 140 cases, who had no signs or symptoms of vitamin A deficiency, 84, i e 60 per cent, were normal as regards dark adaptation Consequently, it is reasonable to assume that these conjunctival signs do not indicate a deficiency of vitamin A Most observers have mentioned these conjunctival manifestations as signs of vitamin A deficiency so their incidence

in definite cases of vitamin A deficiency, e g nutritional nightblindness and keratomalacia, is of interest (Table VIII) —

TABLE VIII

Incidence of conjunctival pigmentation, xerosis and Bitot's spots in cases of nightblindness and keratomalacia

Condition	Nightblindness	Keratomalacia
Pigmentation only	36	50
Xerosis only	Nil	13
Pigmentation and xerosis	19	120
Pigmentation, xerosis and Bitot's spots	21	8
None of above signs	15	36
TOTAL	91	227

In cases of nightblindness, 83 5 per cent and in cases of keratomalacia, 84 2 per cent had one or more of these signs The findings of previous observers were therefore fairly correct It may be argued that the cases in this group had suffered from vitamin A deficiency of short duration and these signs take a long time to disappear None of the 398 cases gave any history of nightblindness acquired recently or in the past

GROUP III —*Diminished sensitivity of the epithelium of the cornea with or without any manifestations in the conjunctiva*

This group comprises the pre-xerosis and the xerotic stages and is the earliest stage of keratomalacia In the pre-xerosis stage as described by Pillat, there is slight loss of lustre of the cornea, less brilliant 'window reflex' on the cornea and a very slight haze and dryness in the superficial layers These increase on the cornea being exposed to air, the sensitivity of the cornea is diminished and is most marked in the centre and the upper part This is tested by touching the cornea with a piece of cotton-wool In the xerotic stage, the cornea becomes dry and lustreless and the sensitivity is diminished to such an extent that the cornea can be touched without causing blinking of the eyelids Patches of light grey areas appear in the epithelium, most marked in the palpebral fissure The areas are triangular in shape with their base towards the limbus, they may also appear as round, irregular small dots The epithelium is intact With proper treatment these areas completely clear up leaving a completely normal epithelium

GROUP IV —*Keratomalacia without breaking down of the cornea*

In this more advanced stage, the epithelium is still intact but areas of infiltration or degeneration appear in the deeper layers of the cornea These areas are grey in colour with diffuse margins and appear usually in the centre Occasionally, when they are at the periphery, they are crescentric in shape with a small rim of clear cornea between them and the limbus The cornea is dry and lustreless, but with early and correct treatment it completely returns to normal

GROUP V —*Keratomalacia with breaking down of the cornea*

When the cornea begins to break down then it rapidly does so both in depth and on the surface Unless the condition is controlled quickly, it involves the whole of the cornea, perforation takes place and the iris prolapses Even with early treatment some permanent damage to the cornea results, which may be either a leucoma or an adherent leucoma or a partial anterior staphyloma The presence of infection in the conjunctival sac is of great importance in this stage, because if the conjunctiva is infected, a rapid spread of the ulcer with even perforation of the cornea takes place and the eye is lost from panophthalmitis In the absence of infection, notwithstanding gross changes in the cornea, there is no reaction, i e no ciliary injection or injection of the conjunctival blood vessels, the chief symptoms being sensitiveness to light and watering of the eye

GROUP VI —*The stage of keratomalacia in which the eye is completely lost.*

This takes place either by atrophy resulting in phthisis bulbi or an anterior staphyloma is formed If infection occurs, panophthalmitis results

General condition in keratomalacia —The general condition of a child in keratomalacia is low The child is emaciated with a distended abdomen and dry, brittle, scanty hair The mouth and tongue are dry The child is irritable and cries a great deal The skin is loose, dry and dark in colour Diarrhœa is often present in advanced cases and may be the cause or the effect of the vitamin A deficiency

As regards the prevention of blindness groups III and IV are the most important, since in these stages, with proper general treatment, the majority of the cases can be completely cured without causing any defect of vision

Distribution of cases according to the different groups —Table IX shows the distribution of keratomalacia cases Group III—III indicates that both eyes were in group III and so on

TABLE IX

Distribution of cases according to groups.

Group	Number	Group	Number
III—III	31	IV—V	29
III—IV	13	IV—VI	4
III—V	4	V—V	63
III—VI	3	V—VI	26
IV—IV	36	VI—VI	18
	TOTAL 227		

Table IX shows that 80 cases only attended the hospital in the early stage, namely groups III—III, III—IV and IV—IV, and 89 cases when the condition was almost hopeless, i e in groups V—V and V—VI, and that 18 cases were already blind, that is group VI—VI

Sex incidence —The distribution according to sex in keratomalacia was as follows Of the total number of cases, 145, i e 63 9 per cent, were males and 82, i e 36 1 per cent, were females This agrees with the proportion of males and females in the Calcutta population

which is 2 1 This proportion stands out in contrast to that observed in the case of hæmeralopia Keratomalacia is essentially a children's disease, but in the age groups over 10 years in keratomalacia cases the proportion of males to females is still maintained at 2 1 As women are reluctant to come to hospital, it is probable that the incidence of keratomalacia is higher amongst them

Age group—The age distribution of keratomalacia cases is given in Table X —

TABLE X

Age distribution of keratomalacia cases (not recorded in 2 cases)

	Below 3 months	Below 6 months	Below 9 months	Below 1 year	Below 5 years	Below 10 years	Below 20 years	Below 30 years	Below 40 years	Over 40 years	TOTAL
Number of cases	24	23	13	24	92	20	13	10	3	3	225
Percentage	10 7	10 2	5 8	10 7	40 9	8 9	5 8	4 4	1 3	1 3	100

One hundred and seventy-six, i e 78 3 per cent, of the cases were below 5 years of age and of these 84 were infants below the age of 1 year The annual birth rate in Calcutta is about 40,000, according to the 1931 Census Since there are many other hospitals and facilities for treatment, it is apparent that the incidence of the disease amongst infants is high and calls for special attention

Community—Of the two hundred and twenty-seven cases of keratomalacia, 163 were Hindus, 59 Mohammedans and 3 Christians The ratio of Hindus to Mohammedans is 2 8 1 This agrees with their proportion in the population of Calcutta As the Mohammedans do not attend the hospital in as large numbers as the Hindus, it appears that the incidence of keratomalacia is higher amongst the former

Diarrhœa in keratomalacia cases—Diarrhœa may cause vitamin A deficiency by interfering with the absorption of fat and vitamin A deficiency may cause diarrhœa due to metaplasia, hyperplasia and ultimately keratinization of the gastro-intestinal epithelium

Out of 227 cases examined, 53 cases had diarrhœa The duration of the diarrhœa was as follows less than 2 weeks, 4, less than 3 weeks, 7, less than 4 weeks, 6, less than 2 months, 11, less than 3 months, 7, over 3 months, 1 Five were early cases of keratomalacia, the remainder were advanced or very advanced cases All were treated with parenteral administration of 'Prepalin' Thirty-two showed improvement both as regards the general as well as the ocular conditions, 3 showed no improvement and the rest did not come to hospital again

Jaundice in keratomalacia cases—Sixteen cases had jaundice of the obstructive type, giving an immediate direct positive van den Bergh reaction The majority of them did not know that they had jaundice In 5 cases, the duration was as follows 1 month, 1, 2 months, 1, 4 months, 1, 5 months, 1, 1½ years, 1 All these cases were treated with parenteral administration of 'Prepalin' and one case with 'vitamin A concentrate', 9 cases improved and 7 cases failed to attend hospital again

Adult keratomalacia—Sixteen cases of keratomalacia were recorded over 20 years of age Three cases were due to jaundice and 2 cases were due to gastro-intestinal disturbances

Treatment of keratomalacia —Out of 227 cases, 210 were treated and 17 cases failed to attend the hospital The results of treatment are given below in Tables XI-A to XI-D —

TABLE XI-A

Result of treatment in keratomalacia, early cases, clinical groups III—III, IV—IV and III—IV

Treatment	Improvement		Duration of treatment			
	No	Yes	Less than 1 week	Less than 2 weeks	Less than 1 month	Failed to attend
Cod liver oil, 17 cases	*Nil*	9	2	5	2	8
Shark liver oil, 10 cases	*Nil*	8	*Nil*	5	3	2
'Prepalin', 50 cases	*Nil*	39	23	12	4	11
Vitamin A concentrate, 1 case	*Nil*	1	*Nil*	1	*Nil*	*Nil*
78 cases		57	25	23	9	21

TABLE XI-B

Result of treatment in keratomalacia, moderately advanced cases, clinical groups III—V and III—VI

Treatment	Improvement		Duration of treatment			
	No	Yes	Less than 1 week	Less than 2 weeks	Less than 1 month	Failed to attend
Cod liver oil, 3 cases	*Nil*	2	1	1	*Nil*	1
'Prepalin', 4 cases	*Nil*	3	2	1	*Nil*	1
7 cases		5	3	2		2

TABLE XI-C

Results of treatment in keratomalacia, advanced cases, clinical groups IV—V and IV—VI

Treatment	Improvement		Duration of treatment			
	No	Yes	Less than 1 week	Less than 2 weeks	Less than 1 month	Failed to attend
Cod liver oil, 2 cases	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	2
Shark liver oil, 1 case	*Nil*	*Nil*	*Nil*	*Nil*	*Nil*	1
'Prepalin', 29 cases	1	12	4	4	4	16
32 cases	1	12	4	4	4	19

TABLE XI-D

Result of treatment in keratomalacia, very advanced cases, clinical groups V—V, V—VI and VI—VI

Treatment	Improvement		Duration of treatment			
	No	Yes	Less than 1 week	Less than 2 weeks	Less than 1 month	Failed to attend
Cod liver oil, 15 cases	1	3	Nil	1	3	11
Shark liver oil, 15 cases	1	6	2	2	3	8
Preparations, 63 cases	2	38	22	11	7	23
93 cases	4	47	24	14	13	42

Relation of the condition of the skin to vitamin A deficiency

Pillat (1929), Frazer and Hu (1931) in China and Lowenthal (1933) in Africa described a follicular hyperkeratosis of the skin in cases of vitamin A deficiency Nichols (1933) gave this condition the name 'phrynoderma' He considered it as a sign of vitamin A deficiency

In this series out of 91 cases of nutritional nightblindness, there were 2 cases of phrynoderma i e 2 2 per cent and out of 227 cases of keratomalacia, there were 10 cases of phrynoderma i e 4 4 per cent In 140 cases of the control group no phrynoderma was noted

Three cases of phrynoderma from the Skin Department of the Medical College, Calcutta, without any other sign or symptom of vitamin A deficiency, were examined with the biophotometer Two of them were normal and in the other case biophotometric examination could not be done

SUMMARY

1 Conjunctival pigmentation, either alone or in combination with xerosis or Bitot's spots is not necessarily a sign of vitamin A deficiency

2 Keratomalacia is most common in children below 5 years of age About 10 5 per 10,000 of infants suffer from keratomalacia in Calcutta

3 Vitamin A by parenteral administration is the quickest and most effective way of treating the disease This method is especially indicated in cases associated with diarrhœa or jaundice

ACKNOWLEDGMENTS

Our thanks are due to Professor G Sankaran, Professor of Biochemistry, All-India Institute of Hygiene and Public Health, Calcutta, and the staff of the Eye Infirmary, Medical College Hospitals, Calcutta, for their co-operation in this investigation, also to Dr C Chandra Sekar, Assistant Professor of Vital Statistics, All-India Institute of Hygiene and Public Health, Calcutta for his valuable help in the statistical interpretation

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LUFFA ACUTANGULA THE CHEMICAL AND PHARMACOLOGICAL INVESTIGATION OF *LUFFA* SEEDS

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[Received for publication, September 28, 1942]

POWDERED kernels of the seeds of *Luffa acutangula* in doses of 5 to 30 grains are used by physicians practising indigenous systems of medicine as an emetic and an expectorant The seeds of *luffa* are very cheap No investigation of its chemistry and pharmacology has been undertaken It was considered desirable to investigate the matter The present investigation deals with the chemistry and pharmacology of the seeds of *Luffa acutangula*

The unripe fruits of *Luffa acutangula* are commonly eaten as a vegetable but the ripe seeds are very bitter The chemical investigation has shown that the seeds contain a fixed oil, a saponin glucoside and an enzyme

I The fixed oil

The oil was extracted with petroleum ether The average yield of the oil was 47 per cent of the kernels and calculated in terms of the seeds it was 23 per cent The physical and chemical characteristics of the oil were as follows —

(*a*) *Physical* —

Density at $\frac{15\cdot5°C}{15\cdot5°C}$		0 9212
Refractive index η_D^{20}		1 4695
Specific refractive power		0 5116
Viscosity (as compared with water at 20°C) at	20°C	0 554
	26°C	0 409
	40°C	0 329
	60°C	0 132
Specific temperature reactions		87 8

(*b*) *Chemical* —

Acid value	2 5
Saponification value	196 5 to 197 5
Unsaponifiable matter	1 67 to 1 7 per cent
Reichert Meissl number	0 392
Hehner number	92 0 per cent
Acetyl number	12 2
Mixed fatty acid melting point	38°C
Iodine value	5 1

II The saponin glucoside

The saponin glucoside is present to the extent of 2 per cent of the seed The fat-free powder of the kernel of *Luffa acutangula*, dried in a current of warm air to remove the petroleum ether, was extracted with 90 per cent ethyl alcohol in a Soxhlet apparatus The alcohol was removed under vacuum The residue, after treating with sulphuric ether to remove the last traces of the fat and green colouring matter, was refluxed twice with ethyl acetate for 24 hours to remove any gummy substances It was dried in a vacuum desiccator for a week, dissolved in methyl alcohol and refluxed for half an hour with addition of a little animal charcoal and

filtered From this the saponin was precipitated with absolute ethyl alcohol and dried over calcium chloride in vacuum

It is an amorphous powder, bitter in taste, soluble in water, methyl alcohol and 90 per cent ethyl alcohol It is slightly soluble in absolute alcohol but is insoluble in ether, benzene, chloroform and ethyl acetate It is optically active being dextro-rotatory $[\alpha]_D^{20} = \pm 28.6$ and melts at 190°C to 195°C Its acetyl derivative melts at 150°C On shaking, it gives persistent froth On heating with dilute hydrochloric acid it is hydrolysed into an insoluble substance melting at 164°C and a reducing sugar, which readily decolorizes bromine water, gives red coloration on warming for some time with resorcinol in hydrochloric acid, thus showing the presence of aldohexoses The melting point of the osozone of the reducing sugar was 198°C to 200°C The saponin is easily reduced by emulsin and more readily so by the enzyme prepared by us from the seeds of *luffa*

It gives the following further reactions —

(1) It is weakly acid to litmus (2) It is precipitated by neutral lead acetate (3) Basic lead acetate gives no precipitate (4) It gives violet coloration with concentrated sulphuric acid (5) It gives violet coloration with equal parts of ethyl alcohol and sulphuric acid and a drop of ferric chloride (6) The mixture of sodium nitrate and the saponin glucoside gives no coloration with a drop of sulphuric acid (7) It gives yellowish brown colour with Nessler's reagent

III The enzyme

The method of R Willstatter and W Csányi for emulsin, as described by Rosenthaler (1930), was used for the extraction of the *luffa* enzyme

The enzyme is a white powder and its action on *luffa* saponin glucoside and on salicin was compared with that of emulsin The results are given below in the Table —

TABLE

The comparative rates of hydrolysis of luffa *glucoside by* luffa *enzyme and emulsin and that of salicin by* luffa *enzyme and emulsin*

Time in hours	GLUCOSIDES								
	Luffa GLUCOSIDE 0 5 PER CENT SOLUTION						SALICIN 0 25 PER CENT SOLUTION		
	ENZYMES						ENZYMES		
	Nil		*Luffa*		Emulsin		*Nil*	*Luffa*	Emulsin
	39°C	45°C	39°C	45°C	39°C	45°C	39°C	39°C	39°C
	Mg per 100 c c calculated as glucose								
0	2	2	2	2	2	2	*Nil*	*Nil*	*Nil*
½			5	7	3	5		8	27
1	2	2	20	20	14	14		12	70
2		3	35	34	17	15	*Nil*	17	110
3		3		49		17		25	127
4		3	54	58	20	19	*Nil*	27	129
24	10		161		94		5	87	144
48	12		175		113				
72	15		184		119				

Note —Five mg of each of the enzymes were added to 5 c c of the solutions

Pharmacological Investigation

Toxicity —The toxicity of the saponin was studied on frogs The minimum lethal dose (*m l d*) of the glucoside for frogs (*Rana tigrana*) was determined by injecting 0 4 to 1 per cent solution in the ventral lymph sac

The *m l d* for frogs was found to be 0 2 g per kilogram of body-weight

Hæmolysis —The hæmolytic effects of the *luffa* saponin were studied on dog's washed red blood corpuscles suspended in an isotonic buffered Ringer solution Solution of saponin (Merck) was used as control The hæmolytic effects of *luffa* saponin are quite comparable to that of Merck s saponin It causes complete hæmolysis of r b c in an hour in 1 35,000 dilution whereas the Merck s saponin causes complete hæmolysis in 1 40 000

Frog s heart —The perfusion of frogs' hearts showed a digitalis-like action in concentration of 1 in 1,000 in 2 hours

Dog s heart —Intravenous injection of the glucoside in dogs produced slowing of the heart with a slight rise in blood pressure, but even 1 g of the saponin glucoside did not produce systolic stand The action was considered of no therapeutic importance

Alcoholic extract —The effects of alcoholic extract were studied on dogs One in 1 alcoholic extract of the seeds was prepared by percolation and concentration under reduced pressure Alcohol was evaporated before administration of the drug

In doses of 1 c c per kg in dogs it caused death Post-mortem examination showed extreme irritation of the intestinal tract especially the small intestine There was no actual ulceration In 0 5 c c per kg it caused vomiting and diarrhœa, in 0 25 c c per kg it caused sometimes purgation and vomiting but always caused great deal of salivation

Aqueous extract —The fresh kernels rubbed in water were given to dogs by mouth 0 5 g per kg caused salivation and vomiting in about an hour and 1 g per kg caused vomiting from 15 minutes to an hour

Oil —The effect of oil was studied in dogs Five c c to 12 c c of the oil given to dogs by stomach-tube caused purgation and vomiting and in some cases this was accompanied by blood When given intramuscularly (2 c c to 6 c c) it caused locally swelling of the part, the swelling remained for a number of days depending upon the quantity of the oil given

Discussion

The fruit of *Luffa acutangula* is used in Ayurveda as an anthelmintic, stomachic, antipyretic, is said to cure biliousness, asthma, bronchitis and flatulence It is also used in Yunani medicine for similar purposes (Kirtikar and Basu, 1933)

The pharmacological findings of the present investigation show the drug to be an irritant of the gastro-intestinal tract The alcoholic extract of seeds caused irritation of the intestinal tract, especially the small intestine As small a dose as 0 25 g per kg of the seed in alcohol (1 1) caused vomiting and diarrhœa in dogs The oil has a similar effect on the intestine

The crushed kernels also cause vomiting but are not so effective as the alcoholic extract The drug is very cheap and, if clinical trial is given, it may be possible to find a cheap expectorant

Summary

1 The chemistry and pharmacology of the *luffa* seed have been studied

2 It contains a fixed oil, a saponin and an enzyme

3 It causes vomiting and purging in dogs In small doses it causes nausea and salivation

4 Its use in indigenous medicine as an expectorant and emetic has a rational basis It is a very bitter substance and is worth a clinical trial in human beings

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THE PHARMACOLOGICAL ACTION ON THE CIRCULATORY SYSTEM OF A BITTER PRINCIPLE ISOLATED FROM *SECURIGER A SECURIDACA* (LINN) DAGEN *ET* DORFLER (N O LEGUMINOSÆ)

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[Received for publication, October 26, 1942]

INTRODUCTORY

THE plant *Securigera securidaca* (Linn) Dagen *et* Dörfler belongs to the Natural Order Leguminosæ It is found in Syria, Palestine, Dalmatia, Germany and France It can be brought under cultivation in the plains of India Its seeds are sold in the Indian market as diabetes seeds and are used by 'Hakims' here as a remedy for diabetes Interest was created in a scientific study of the active constituents of this drug on the report that administration of the seeds gave rise to circulatory disturbances in some of the cases The presence of a potent principle or principles was considered probable and it seemed worth while investigating its properties on the circulatory system and on blood-sugar The present paper deals with circulatory effects The action on blood sugar will form the subject of a later communication

CHEMISTRY

The crushed seeds were extracted with petroleum ether to remove the oil The extracted seeds were re-extracted with chloroform The chloroform solution was concentrated to a small bulk and was shown not to contain any alkaloid or glucoside A bitter principle was precipitated from the concentrated product by petroleum ether The white powder obtained was dissolved in chloroform and again precipitated by petroleum ether Finally, it was dissolved in a small quantity of methyl alcohol and allowed to crystallize The crystalline bitter principle was soluble in alcohol, chloroform, ethyl acetate and was sparingly soluble in cold and hot boiling water

EXPERIMENTAL

For the present work, the crystalline bitter principle was always employed Cats were used in all the experiments and the bitter principle was administered through the femoral vein in 0 05 per cent solution, unless otherwise stated

Effects on blood-pressure —A rise of blood-pressure which varied from 30 mm to 50 mm of mercury was obtained on administration of 0 05 mg to 0 075 mg of the bitter principle per kilo body-weight of urethanized or decerebrated cats (Graph 2) In some of these animals this rise was followed by a slight fall of blood-pressure To ascertain the mechanism which was responsible for the pressor effect the bitter principle was administered in the same doses on spinal preparations and on animals whose parasympathetic endings were completely paralysed with atropine In all these animals the pressor effect was very much augmented, and these stood much bigger doses than the urethanized animals Furthermore, it was observed that pilocarpine produced its usual effect after administration of the bitter principle It might be concluded from these that the pressor effect of the drug was not due to its paralysing action on the vagal system

The drug was then administered on spinal cat in which the vasomotor nerve-endings were completely paralysed with repeated injections of ergotoxine phosphate It was noticed that the response of the drug after ergotoxine was much smaller than the response obtained with the same dose of the drug before administration of ergotoxine (Graph 5) This showed that the rise of blood-pressure was at least partly attributable to its stimulant action on the sympathetic endings The short and sharp rise of pressure noted with the drug after ergotoxine was possibly caused by its direct action on the blood vessels or on the cardiac musculature

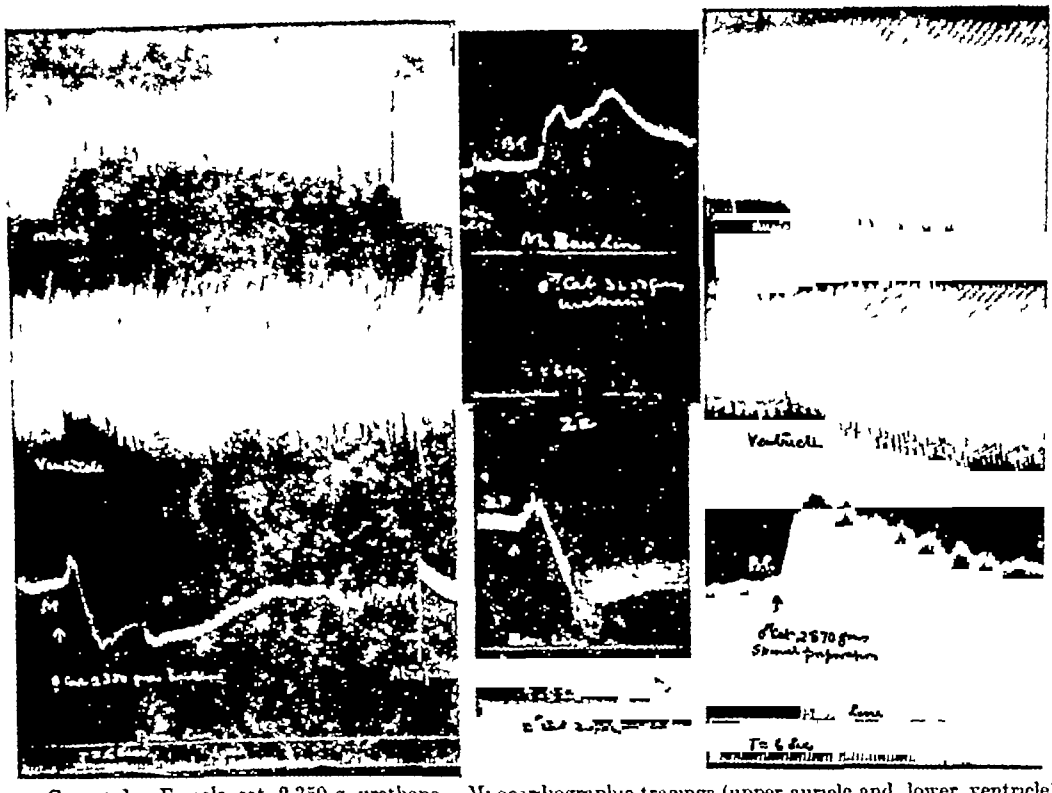
To elicit if the bitter principle had any direct action on the blood vessel recourse was taken to the method of cocainization described by Tainter and Chang (1927) and Tainter (1929) Sixteen milligrams of cocaine per kilo body-weight were injected subcutaneously after 1 mg of atropine per kilo body-weight in urethanized cats The bitter principle administered to these animals produced a weak response in most of these animals (de-sensitization) It can, therefore, be concluded that the drug acted also partly on the plain muscles of the blood vessels to produce the pressor effect

The bitter principle when administered in doses varying from 0 1 mg to 0 15 mg per kilo body-weight in urethanized animals produced a short rise of blood-pressure which was followed by a fall with slowing of the rate of the heart (Graph 2-*a*) In some cases the fall of pressure varied from 10 mm to 40 mm of mercury or more and lasted for about 2 to 5 minutes or even longer The same effects were also observed in the decerebrated preparations but not in the spinal animals In the decerebrated and urethanized animals the blood-pressure became irregular in some cases It has been pointed out that in spinal preparations not only there was no fall of blood-pressure but the rise was more marked and persistent, which in some cases varied from 50 mm to 90 mm of mercury and lasted from 5 to 18 minutes The fall of blood-pressure with slowing of the heart which followed the short rise of blood-pressure after bigger doses of the bitter principle might partly be a reflex action attributable to the rise of pressure in the sinus caroticus but it was mainly due to stimulation of the vagus centre in the medulla, because, firstly, it disappeared in the spinal animals in which the vagus centre was removed and, secondly, the fall of pressure and slowing of the heart were persistent and were out of proportion to the effect which might be expected from the amount of rise observed in those cases, in some cases there being a marked fall following an insignificant rise

Effects on myocardium —In myocardiographic experiments on spinal animals the drug [illegible] very small doses produced no effect either on the auricles or on the ventricles, but with doses varying from 0 05 mg to 0 15 mg per kilo body-weight the amplitude of contraction of the auricles and ventricles was increased with the rise in blood-pressure This effect on the auricles and ventricles lasted for a very long time (Graph 3) It was also observed that on administration of similar doses of the bitter principle on atropinized animals the effects on the auricles, ventricles and blood-pressure were very markedly increased

In the urethanized cats, with doses varying from 0 1 mg to 0 15 mg per kilo body-weight, the auricles went into a state of dilatation and their amplitudes of contraction and relaxation were markedly reduced and the rhythm of the heart was slowed simultaneously with the fall of blood-pressure These animals also showed irregularities of blood-pressure An injection of atropine at this stage relieved these irregularities and the auricles and the ventricles began to beat regularly (Graph 1)

Effects on isolated heart —The isolated hearts of kitten were fed through the coronary arteries with different dilutions of this bitter principle in oxygenated Locke's solution having a pH of 7 2 and temperature of 37 5°C Up to and above 1 in 150,000 dilutions it practically produced no effect on the heart In dilution of 1 in 50,000 there was a marked increase in the amplitude of contraction but the frequency of heart was increased only for a short time, roughly for about 1 minute The increase in the amplitude of contraction used to last for a fairly long time, in some cases even exceeding 5 minutes (Graph 4) The outflow of blood through the coronaries as studied from the total output from the cut pulmonary artery was reduced steadily In one case it was reduced from 30 c c per minute to 24 c c within 2 minutes and in 5 minutes it was reduced to 12 c c per minute When bigger doses were given to this heart the coronary outflow was still further reduced and the heart became irregular and ultimately the ventricles failed to respond to every beat of the auricles, thus inducing a condition of heart-block The irregularity of the heart observed with this bitter principle disappeared after administration of atropine Further, it was observed that on administration of smaller doses of this bitter principle after injection of atropine, the heart showed practically the same increase in the amplitude of contraction, thus showing that the muscular action of the bitter principle on the heart with smaller doses overshadows the vagal effects of the drug



GRAPH 1.—Female cat, 2 350 g urethane Myocardiographic tracings (upper auricle and lower ventricle) and carotid blood pressure Downstrokes systole and upstrokes diastole Shows the effects of injection of 0 1 mg of the bitter principle of *Securigera securidaca* per kilo body weight At second arrow 1 mg of atropine was given intravenously and the effects shown after 5 minutes Time, 6 seconds

GRAPH 2.—Male cat 3 250 g urethane Carotid blood pressure Shows the effect of injection of 0·075 mg of the bitter principle of *Securigera securidaca* per kilo body weight Time 6 seconds

GRAPH 2-*a*.—Male cat 2 710 g urethane Carotid blood pressure Shows the effect of injection of 0 12 mg of the bitter principle of *Securigera securidaca* per kilo body weight Time, 6 seconds

GRAPH 3.—Male cat 2,870 g spinal preparation Myocardiographic tracings (upper auricle and lower ventricle) and carotid blood pressure Downstrokes systole and upstrokes diastole Shows the effect of injection of 0 1 mg of the bitter principle of *Securigera securidaca* per kilo body weight Time, 6 seconds

GRAPH 4.—Perfusion of isolated heart of kitten Downstrokes systole and upstrokes diastole Shows the effect of 1 in 50 000 solution of bitter principle of *Securigera securidaca* At second arrow atropine was added to the perfusion fluid Time 3 seconds

GRAPH 5-*a*, *b*, *c*, *d*.—Male cat 2 850 g spinal preparation *a* *b* and *c* show the effects on blood pressure of injection of 0 14 mg of the bitter principle of *Securigera securidaca* per kilo body weight, before injection of ergotoxine sufficient to paralyse the vasomotor nerve-endings *d* shows the effect of the same dose of the bitter principle after ergotoxine Intervals between *a*, *b* and *c* are 6 minutes each Time, 6 seconds

Effect on the volume of organs —The volumes of the spleen and kidney were reduced on intravenous injection of the drug which corresponded with the rise of blood-pressure

Discussion

The bitter principle produced its pressor effect by acting partly on the sympathetic and partly on the plain muscle of the blood vessels The augmentation of the heart-beat observed in the myocardiograph experiments and in the isolated hearts also greatly contributed to the rise of blood-pressure The reduction of the coronary outflow which accompanied the augmentation of the heart-beat with transient acceleration or without any acceleration at all can only be explained by the direct stimulant action of the drug on the muscle overshadowing the effect of stimulation of the sympathetic

The fall of blood-pressure and slowing of the rate of the heart observed with the bitter principle in bigger doses on animals anæsthetized with urethane and in the decerebrated animals and its absence in cats which had been given sufficient doses of atropine to paralyse the parasympathetic endings pointed to its vagal origin The cardiac musculature was never depressed and the experiments with volume changes in organs showed no signs of dilatation of the blood vessels Further, the complete disappearance of the depressor effect in the spinal animals conclusively showed that the depression was due to stimulation of the vagal centre in the medulla

The isolated heart fed through the coronaries showed that in bigger doses the heart became irregular and a condition of heart-block was induced It is known that on stimulation of vagus various effects are produced which are chiefly due to the difference in the place affected When the fibres of the vagus which are distributed to the remains of the sinus are stimulated we get a slowing which affects the whole heart, whereas when those fibres which supply the A V bundle are stimulated its most pronounced effect will be on the propagation of the excitatory process from the auricles to the ventricles and a condition of block will be produced The heart-block that was induced with bigger doses of the bitter principle in the isolated heart is thus attributable to the excitation of vagal fibres distributed to the A V bundle

From a study of the effects on the circulatory system observed with this bitter principle, one can easily understand that a principle which has such a pronounced effect on the circulation will produce some symptoms or other with adequate doses

Summary and conclusions

1 The bitter principle isolated from *Securigera securidaca* produces its pressor effect on circulation by its direct action on the cardiac musculature and plain muscle of the blood vessels and also partly by its action on the sympathetic nerve-endings

2 The fall of blood-pressure observed with bigger doses of the bitter principle is due to the stimulation of the vagal centre in the medulla

Acknowledgments

The seeds used in this investigation were kindly supplied by Dr R C Sen of Calcutta The plants were identified by Mr S N Bal, Officer-in-Charge, Botanical Survey of India, Indian Museum, Calcutta The chemical investigation of the seeds was carried out in the Department of Chemistry of the School of Tropical Medicine, Calcutta, by Professor S Ghosh and Mr N N Ghosh I am indebted to all of them for the help they have rendered I express my indebtedness to Dr B Mukerji for some suggestions he gave while writing this paper I am also indebted to my assistants for the help rendered by them at my experiments

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HYPNOTIC EFFECT OF *RAUWOLFIA SERPENTINA* THE PRINCIPLE UNDERLYING THIS ACTION, ITS PROBABLE NATURE

BY

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[Received for publication, November 26, 1942]

IN spite of the popular use of *Rauwolfia serpentina* in Bihar and the United Provinces as a sedative remedy for insanity (Pagal-ki-dawai), the hypnotic effect of the drug was not known to the ancient Ayurvedic medicine Chopra *et al* (1933) obtained depression of the central nervous system in both poikilotherm and homœotherm animals from *ajmaline*, the only alkaloid that was isolated at that time from the plant Since then an alcoholic extract of the root has been used by the senior authors at the Carmichael Hospital for Tropical Diseases, Calcutta, as a sedative drug in essential hypertension and insanity but the principle or principles responsible for this action were not definitely known Besides *ajmaline*, two more alkaloids, *serpentine* and *serpentinine*, were isolated by Siddiqui and Siddiqui (1932) A comparative study of the relative toxicity of all the three alkaloids was made by Chopra and Chakravarti (1941) which revealed that neither *ajmaline* nor *serpentine* produced any sedative effect on the central nervous system of white rats, they were, on the contrary, powerful convulsant poisons *Serpentinine*, however, behaved like a mild depressant to the central nervous system in a certain number of experiments These discordant findings of the clinic and the laboratory made us review the problem on a wider experimental basis with a view to ascertain whether the plant possessed any hypnotic principle at all and, if so, what its probable nature could be

EXPERIMENTAL

Investigations were carried out on albino rats between 100 g and 150 g of body-weight Different principles of *Rauwolfia serpentina* in a constant volume of liquid were injected intraperitoneally to the animals which were kept at a temperature of 30°C during experimentation and protected from such stimuli as were capable of exciting or depressing the central nervous system

1 *Action of the isolated alkaloids*—By using graded doses of the hydrochlorides of *ajmaline*, *serpentine* and *serpentinine*, it was observed that the *maximum tolerated dose* (*m t d*) and the *minimum lethal dose* (*m l d*) for *serpentine* were 0 07 mg and 0 1 mg respectively per gramme body-weight of the animal, whereas the same for *ajmaline* and *serpentinine* were on an average 0 1 mg and 0 12 mg respectively The margin of safety was therefore low with these alkaloids and all of them behaved like convulsant poisons including *serpentinine* which was found to be a mild depressant by Chopra and Chakravarti (*loc cit*) The combined action of *ajmaline*, *serpentine* and *serpentinine* in proportions present in the plant, i e 0 1 per cent, 0 08 per cent and 0 08 per cent respectively, was also found to be stimulant

The effect of *m l d* of these alkaloids was as follows For half an hour after injection the animals remained relatively quiet This was followed by signs of motor excitability, twitching, restlessness, clonic convulsions and elevation of rectal temperature by 1°C to 2°C This stage lasted for 20 to 30 minutes and was followed by either a gradual recovery or fatal crisis In fatal cases symptoms of anoxæmia, such as quick respiration, air-hunger, staggering, confusion, unconsciousness, respiratory failure and, finally, heart failure, were noticed At autopsy, the right heart was found to be congested and dilated The action resembled that of a medullary stimulant

2 *Action of the alcoholic extract* —Calculated in terms of the total alkaloidal content, the *m t d* and the *m l d* for the extracts of *Rauwolfia serpentina* were found to be 0 05 mg and 0 07 mg respectively per gramme body-weight of the animal Symptoms appeared in the following sequence 10 to 15 minutes after the administration of the extract the animals became somnolent and restful, breathing was deep but slow, corneal reflexes sluggish, and rectal temperature reduced by 1 5°C to 2°C In some cases even narcosis occurred and the animals slept into death within an hour of administration of higher doses The quantity of alcohol contained in the extract did not produce any effect in the control animals Dose for dose the Bihar sample showed slightly greater hypnotic effect than the Dehra Dun variety of *Rauwolfia*, for this latter *m l d* was found to be 0 08 mg per gramme weight of the animal

3 *Action of the total alkaloids* —The hydrochlorides of the total alkaloids obtained from the above-mentioned varieties of *Rauwolfia serpentina* showed hypnotic effect and the *t d* and *m l d* were found to be 0 05 mg and 0 08 mg per gramme body-weight The time of onset of drowsiness corresponded with the size of the doses utilized It occurred within 15 minutes of the injection of a 0 05 mg per gramme body-weight dose and within 10 minutes when the dose was increased to 0 06 mg Neither narcosis nor death was produced Narcosis, however, was obtained with 0 8 mg /g dose and the rats went into deep hypnosis within 7 to 8 minutes of the administration of the drug, followed by unconsciousness, coma and death The duration of hypnosis varied from 1 to 2 hours No great difference in the activity of the total alkaloids of the two varieties of *Rauwolfia* could be elicited but the hypnosis produced by the total alkaloids from the Bihar sample of *Rauwolfia* was found to be more prolonged It is interesting to note that the natural total alkaloids behaved very differently from the laboratory mixture of *ajmaline*, *serpentine* and *serpentinine*, suggesting that the former should contain some other additional principle which produced sedative effect in spite of the stimulating properties of the latter combination

4 *Action of the total alkaloids free from* ajmaline, serpentine *and* serpentinine —The elimination of the convulsant principles from the total alkaloids of *Rauwolfia* was attempted in the Department of Chemistry, School of Tropical Medicine, Calcutta, with a view to separate the sedative fraction from its physiological antagonists and thereby obtain increased hypnotic effect with the remaining portion of the total alkaloids The new fraction provoked hypnosis in 0 05 mg to 0 08 mg per gramme body-weight doses but failed to elicit any superior effect

5 *Physiological relationship between* Rauwolfia *and* picrotoxin —The stimulating and the depressing principles of *Rauwolfia* appeared to be medullary poisons Their effects were therefore tested against a known medullary poison, such as *picrotoxin*, with a view to ascertain the synergistic or antagonistic effect of the principles *vis-à-vis* this potent medullary stimulant and thus to find out their probable site of action It was observed that both the extract and the total alkaloids were capable of diminishing considerably the convulsant action of *picrotoxin* Under their influence the convulsant dose of *picrotoxin* was nearly doubled and the latent period for convulsion prolonged Similarly, the convulsant action of *picrotoxin* with 0 02 mg per gramme body-weight could be effectively controlled by 0 05 mg per gramme of the extract or of the total alkaloids and hypnosis induced in place of convulsion and agitation The isolated alkaloids—*ajmaline*, *serpentine* and *serpentinine*—produced additive effects with *picrotoxin*

TABLE

Showing action of the different fractions of Dehra Dun and Bihar varieties of Rauwolfia serpentina *in albino rats*

Name of fractions		*M l d* mg /g of body weight	*M l d* mg /g of body weight	Nature of action	Rectal temperature	Relationship with *picrotoxin*
Isolated alkaloids	*Ajmaline*	0 10	0 12	Convulsion	Elevated	Synergism
	Serpentine	0 07	0 10	,,	,,	,,
	Serpentinine	0 10	0 12	,,	,,	,,
	Mixture of *ajmaline serpentine* and *serpentinine*	0 09	0 10		,,	,,
Total alkaloids containing *ajmaline, serpentine* and *serpentinine*	Dehra Dun variety	0 05	0 08	Hypnosis	Reduced	Antagonism
	Bihar variety	0 05	0 08	,,	,,	,,
Total alkaloids free from *ajmaline, serpentine* and *serpentinine*	Dehra Dun variety	0 06	0 08	,,	,,	,,
	Bihar variety	0 05	0·08	,,	,,	,,
Alcoholic extract	Dehra Dun variety	0 05	0 08	,,	,,	,,
	Bihar variety	0 05	0 07	,,	,,	,,

DISCUSSION

From the study of the different principles of *R serpentina*, it appears that none of the isolated alkaloids—*ajmaline, serpentine* and *serpentinine*—possesses any sedative properties They are, on the contrary, medullary excitants and produce agitation, convulsion and acceleration of thermogenesis In toxic doses signs of incoordination, anoxæmia and respiratory failure occur *Serpentine* is probably the most toxic of the three alkaloids and its *m l d* corresponds with the *m l d* of *ajmaline* and *serpentinine* This confirms the finding of Chopra and Chakravarti (*loc cit*)

The sedative and hypnotic properties are, however, present in the alcoholic extract and in the total alkaloids obtained from both the varieties of *Rauwolfia*, and drowsiness, narcosis and a fall in the rectal temperature are elicited by them It follows, therefore, that this principle might be of an alkaloidal nature and different from those already known Failing to isolate this principle chemically an attempt was made to eliminate the convulsant principles from the total alkaloids The remaining fraction showed hypnotic effect though not in doses smaller than 0 05 mg per gramme body-weight of the animal It is, however, doubtful whether the elimination of the alkaloids already referred to, was practicable at the present stage without undermining the action of the remaining fraction of the total alkaloids containing the hypnotic principle The hypnotic principle acted antagonistically to the medullary stimulation of *picrotoxin* It would, therefore, appear that this new principle acts as a medullary depressant, whereas *ajmaline, serpentine* and *serpentinine* stimulate the medulla and its respiratory centre They also act on the vasomotor centre as previously reported by the authors (Chopra *et al*, 1942) It is therefore evident that most of the active principles of *Rauwolfia serpentina* are medullary poisons, some stimulating, while others depressing the centres

CONCLUSIONS

1 The alkaloids *ajmaline, serpentine* and *serpentinine* of *Rauwolfia serpentina* are medullary stimulants and provoke convulsion and anoxæmia

2 The sedative and hypnotic properties are present mainly in the alcoholic extract, and in the total alkaloids and in the total alkaloids free from *ajmaline*, *serpentine* and *serpentinine*

3 The hypnotic principle antagonizes the medullary stimulation of *picrotoxin* and has depressant action on the medullary centres

REFERENCES

Chopra, Bose, Gupta and Chopra (1942)	*Ind Jour Med Res*, **30**, p 319
Chopra and Chakravarti (1941)	*Ibid*, **29**, p 763
Chopra, Gupta and Mukerji (1933)	*Ibid*, **21**, p 261
Siddiqui and Siddiqui (1932)	*Jour Ind Chem Soc*, **9**, p 530

STUDIES IN HÆMOLYSIS

BY

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AND

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[Received for publication, October 26, 1942]

THE subject of hæmolytic anæmias has received its due share of attention during the general advance of the last decade in our knowledge of blood disease, to day we have accurate description of many hæmolytic syndromes The work of Ponder, Haden, Bergenhem, and Fahreus, amongst others, has helped to explain the mechanism of erythrolysis under normal conditions and in certain pathological states, particularly congenital hæmolytic icterus (acholuric jaundice)

Experimental hæmolytic anæmia in animals brought about by the injection of heterophilic hæmolytic serum has been produced many times Belfanti and Carbone were apparently the first to carry out this experiment, as early as 1898 (Dameshek and Schwartz, 1938) Most of the earlier work was more concerned with the theories of immunity than with hæmatology The investigations of truly hæmatological interest were those of Dudgeon, Panton and Ross (1909) confirmed by Muir and McNee (1912), which demonstrated that large doses of heterophilic hæmolytic serum produced hæmoglobinæmia and hæmoglobinuria, whereas small doses produced nucleated red cells and microcytes, and that of Banti (1913) who showed that there are two phases of experimental hæmolytic anæmia (i) *hæmolytic*, characterized by diminution in red cell count and hæmoglobin, and (ii) *regenerative*, characterized by the presence of large numbers of reticulocytes Banti (*loc cit*) also demonstrated that there was increased fragility of the red cells, during the progress of the anæmia, but regarded this as development of special 'fragilizing' activity of the organism

In recent years, Dameshek and Schwartz (*loc cit*) have studied the changes produced in the erythrocytes in the guinea-pig injected with varying doses of anti-guinea-pig-cell hæmolytic serum By varying the dosage of hæmolysin, various types of hæmolytic syndrome were produced The red cells of the guinea-pig became spherocytic with increased fragility to hypotonic sodium chloride solution These workers produced spherocytosis, increased erythrocyte fragility, reticulocytosis and a 'pseudomacrocytic' blood picture in the course of their experiments Besides this experiment they showed (Dameshek and Schwartz, *loc cit*) the presence of isohæmolysins of the immune-body type in the serum of three of their cases of acute hæmolytic anæmia They concluded that all hæmolytic syndromes are due to hæmolysins possibly of different types and present in different amounts, functioning slowly in some cases, violently in others Haden (1939), however, comments, 'These observations only show that spherocytosis can be acquired It is very doubtful whether they have any application in congenital hæmolytic icterus The spherocytosis evidently results here from reaction of the cell to injury by hæmolysin'

The present investigation

The present work was undertaken in order to study further these changes in the red cells produced by the action of hæmolysin *in vivo* as part of a study of the mechanism of blackwater fever and as a preliminary measure to testing certain drugs for their anti-hæmolytic properties *in vivo*. The monkey was chosen as it was very near to man hæmatologically, as well as in the scale of evolution, also because quantities of blood adequate for the various hæmatological investigations could easily be obtained by venepuncture from the monkey. Anti-monkey cell hæmolytic sera of good titre could be readily produced in rabbits by repeated injection of washed monkey cells.

Hæmatological investigations were directed mainly to determining the changes in the red cells. The investigations consisted in the determination of the hæmoglobin percentage, the enumeration of mature red cells and of reticulocytes, the measurement of the packed cell volume, and the calculation of mean corpuscular volume (MCV), the mean corpuscular hæmoglobin (MCH), and the mean corpuscular hæmoglobin concentration (MCHC), the measurement of the red cell diameters, and the calculation of the mean cell diameter (MCD), its standard deviation (s. d.), the coefficient of variation, and the calculation of the mean corpuscular average thickness (MCAT) from the MCV and MCD. The test for fragility and the van den Bergh test were done, and the bilirubin content estimated.

The technique followed is given in full detail in 'Hæmatological technique' (Napier and Das Gupta, 1942) but a few points may be mentioned in this connection. The hæmoglobin was estimated with a Hellige hæmometer with coloured glass prisms as the standard, 100 per cent being equal to 13·75 g. of hæmoglobin. Standardized Zeiss pipettes—the same pipette for all successive counts—were used for the red blood cell count. Wintrobe's hæmatocrit tube was used for estimating cell volume. The reticulocyte counts were made according to method B II (Napier and Das Gupta, *loc. cit.*) which is a modification of the method of Osgood and Wilhelm (1934). The fragility of red blood cell was tested by the qualitative method. The red cell diameters were measured by a modification of Hynes and Martin method, which gives reasonably correct results (Napier, Sen Gupta and Chandra Sekar, 1941; Napier and Sen Gupta, 1941). All these investigations were carried out before injection of the hæmolysin and frequently after it, the first count being done usually 4 hours after the injection.

Preparation of anti monkey cell hæmolysin.—Blood was collected by venepuncture from the monkey with a sterile syringe containing 2 c.c. to 3 c.c. of 3·8 per cent sodium citrate in normal saline. The blood citrate mixture was centrifuged and the supernatant fluid removed with sterile pipettes. The cells were washed with three changes of sterile normal saline and finally suspended in normal saline making about 40 per cent suspension. This suspension was injected into the ear veins of four rabbits in each series of experiments. In all, six injections were given to each rabbit, two injections being given every week, the dosage was: the first injection, 1·0 c.c., the second, 1·5 c.c., and the third and subsequent injections, 2·0 c.c. of 40 per cent cell suspension. Blood was collected aseptically from the heart of the rabbits 10 days after the last injection, the serum was separated and heated at 55°C for ½ hour and preserved undiluted in vaccine vials in a refrigerator. Usually fresh serum was used in the experiments.

Titration of the hæmolysin.—The hæmolytic sera from the rabbits were diluted 1/25, 1/50, 1/100, 1/200, 1/400, 1/800, etc. A 3 per cent suspension of washed red cells of monkey was made and a 10 per cent solution of a good titre complement* from guinea pigs. Equal volumes (0·25 c.c.) of each, i.e. hæmolysin, red cell suspension, and complement, were mixed in a series of test tubes and the tubes were incubated in a water bath at 37°C. to 38°C. for 1 hour. The hæmolytic titre (the minimal hæmolytic dose = MHD) was taken to be the greatest dilution at which the red cells showed complete hæmolysis.

For example.—Each tube contains 0·25 c.c. each of dilution of hæmolysin, 3 per cent red cell suspension and 10 per cent complement. As control two tubes are used: (1) containing 0·25 c.c. of red cell suspension and 0·5 c.c. of saline, (2) containing 0·25 c.c. each of red cell suspension, complement and saline. After incubation for 1 hour at 37°C. to 38°C. the results are read as follows:—

\+ complete inhibition
T trace of lysis
± partial lysis and fair amount of red cells
(—) almost complete hæmolysis
— complete hæmolysis

Dilutions of hæmolysin	1/25	1/50	1/100	1/200	1/400	1/800
Reading	—	—	—	—	±	±

Here the titre is 1/200.
The hæmolytic titre obtained in this series of experiments varied from 1/100 to 1/400.

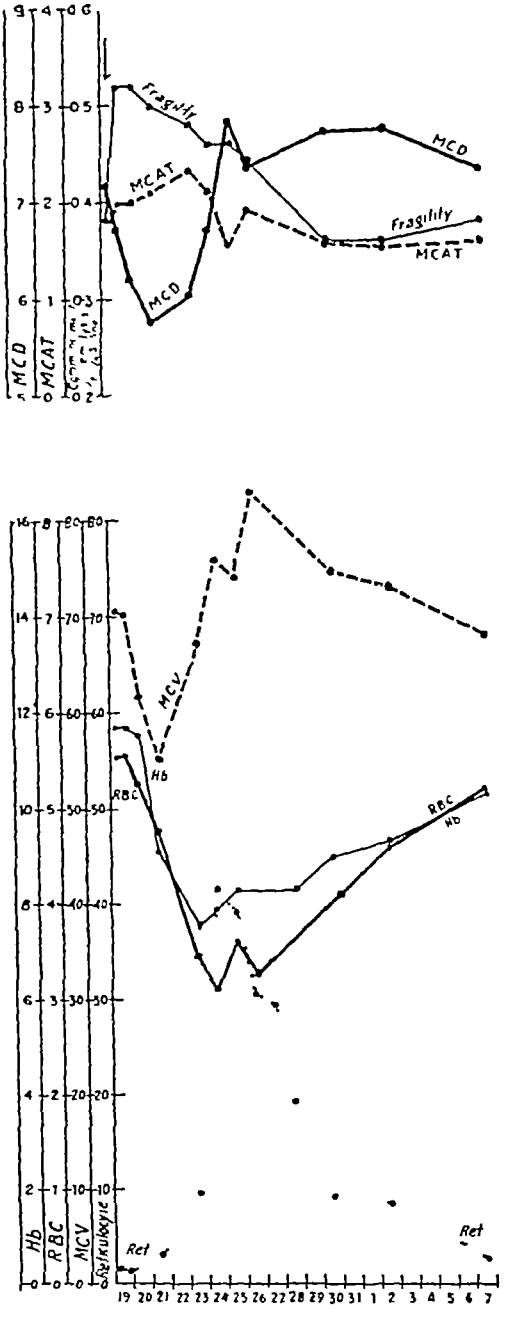
* Obtained through the courtesy of the Imperial Serologist, Calcutta.

I Experiment using single large dose of hæmolysin

In this experiment a single large dose of anti monkey cell hæmolytic serum (100 MHD to 400 MHD) was given intravenously to the monkeys The serum was administered undiluted The effect on the monkeys was noted and the urine repeatedly examined particularly for hæmoglobinuria Examinations were carried out before giving the injection about 4 hours after the injection next day then daily for a few days and, later at longer intervals

GRAPH A

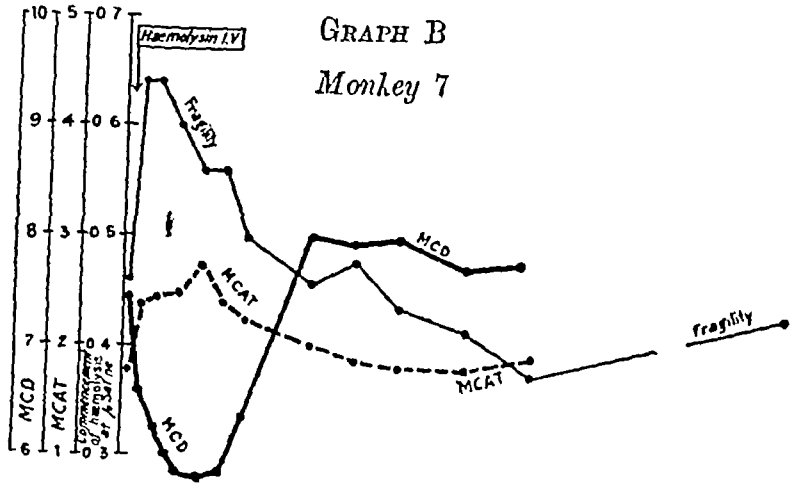
Monkey A



The intravenous injection of the hæmolysin produced a severe reaction in the monkeys There was marked weakness and prostration The monkeys lay on the floor of the cage as if they were seriously ill One of the monkeys had attacks of vomiting The urine that was passed was highly coloured On examination it was found that there was hæmoglobinuria no granular casts or red cells were present The prostration and hæmoglobinuria persisted for the whole day On the next day the monkeys looked more normal and the urine was clear and free from hæmoglobin The usual liveliness was regained in the next 2 or 3 days though the blood picture indicated progressive anæmia The monkeys were kept on a good diet consisting of gram bananas carrots green vegetables, etc , all through the experiment

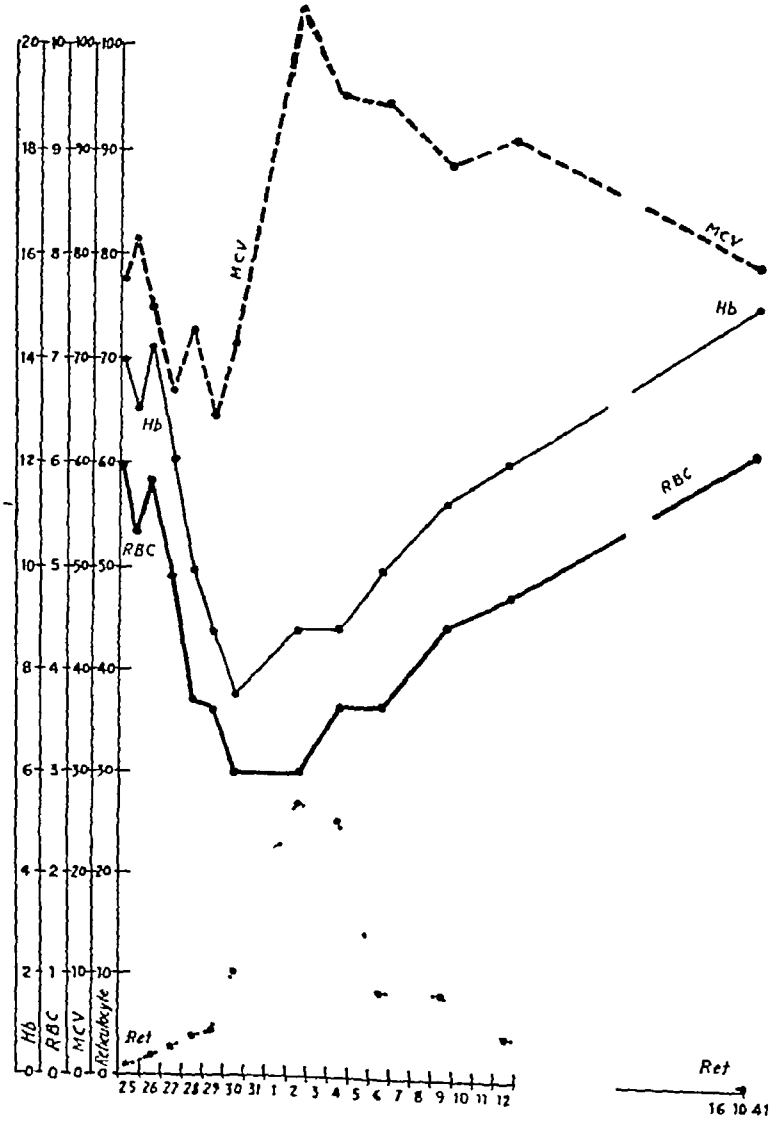
Blood picture (*see* Tables A and B and Graphs A and B) —The percentage of hæmoglobin and the red cell count progressively came down severely up to about the 5th or 6th day after injection, when a reticulocyte 'crisis' occurred and then both hæmoglobin and red cell count went up progressively, until in about a month from the day of injection they reached the previous level The reticulocytes showed a slight rise from the 2nd or 3rd day, the 'crisis' being reached about 5th to 6th day The percentage of reticulocytes then gradually came down The mean corpuscular volume also diminished for the first 3 or 4 days then, as the reticulocytes gradually increased to reach the crisis, the mean cell volume also increased markedly to a level often above that of normal monkeys In the course of the next fortnight the MCV slowly diminished so as to reach the normal level about the end of this period Inspection of the blood smear from day to day showed that there was marked microcytosis during the first 3 or 4 days, thereafter two types of cells could easily be made out, one the microcytes deep-staining and circular in outline,

and the other large cells, the macrocytes, more irregular in outline (*see* Plate I, figs. 1, 2 and 3) The proportion of the cells varied from day to day



GRAPH B

Monkey 7



The study of the series of the Price-Jones' curves (*see* Figs I and II) of the monkeys' red cells during the whole period gave a clearer idea regarding these changes in the red cells The exact changes that took place from day to day in two experimental monkeys are given in detail below as also under the heading 'Statistical Analysis'

The fragility of the red cells to hypotonic saline increases sharply during the day the hæmolysin is administered The blood collected 4 hours after injection of hæmolysin shows a marked increase of fragility (commencement of hæmolysis 0 64 per cent from 0 46 per cent and 0 52 per cent from 0 38 per cent sodium chloride) The increase in fragility persisted for the first 4 or 5 days—though it slowly came down, eventually to below the original level The cells then appear to be more resistant to hypotonic saline than previously for a time, but ultimately the fragility reached the 'normal' level for monkeys

The mean corpuscular average thickness (MCAT) increases sharply soon after the injection It usually remains greater than the normal level during the first 4 to 6 days, then it gradually comes down to a level near about the original value

The van den Bergh test indicated a slight increase in bilirubin content on the day of the injection, but after that there is no indication of any increased hæmolysis

Hæmoglobinæmia was apparent in the sample of blood collected 4 hours after the injection, but in no other subsequent sample

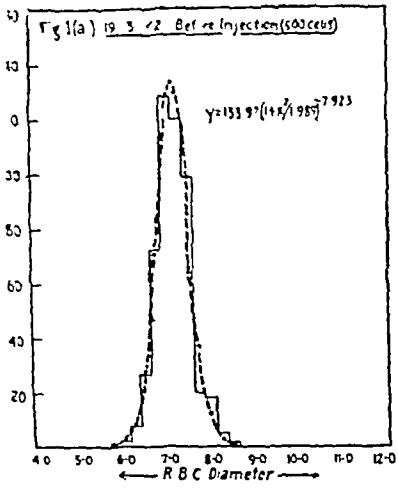


Fig 1(a) 19 3 42 Before Injection(500 cells)
Frequency
R B C Diameter

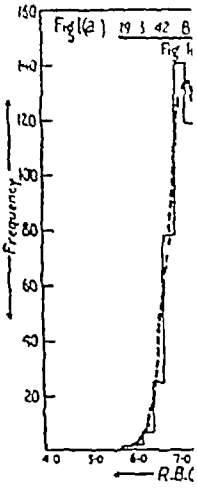


Fig 1(a) 19 3 42 B
Frequency
R.B.C

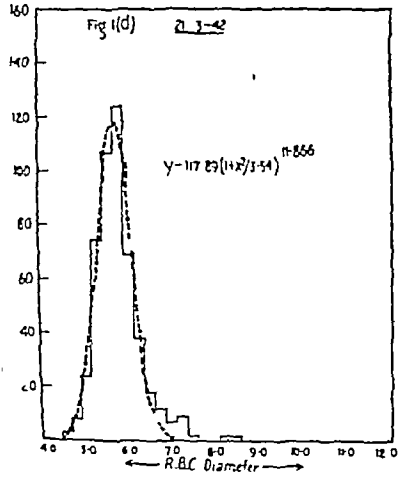


Fig 1(d) 21 3 42
Frequency
R.B.C Diameter

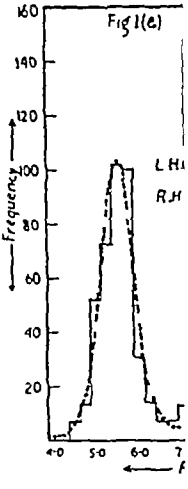


Fig 1(e)
Frequency

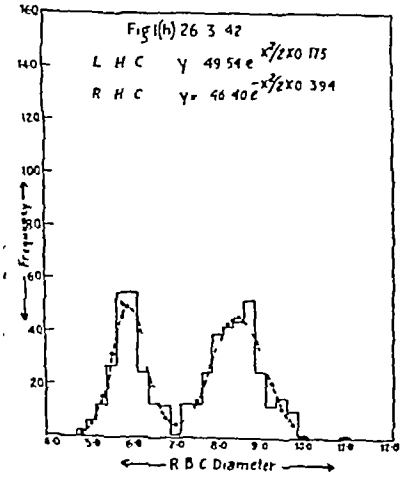


Fig 1(h) 26 3 42
Frequency
R B C Diameter

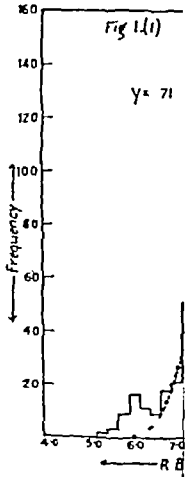


Fig 1.(i)
Frequency

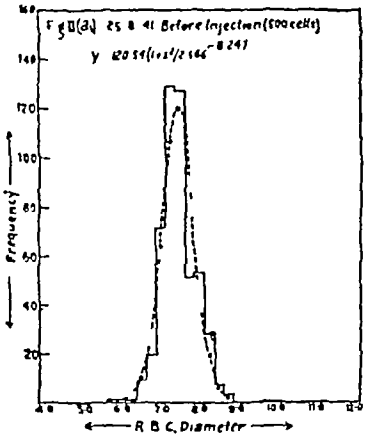


Fig II(a) 25 8 41 Before Injection(500 cells)
Frequency
R B C. Diameter

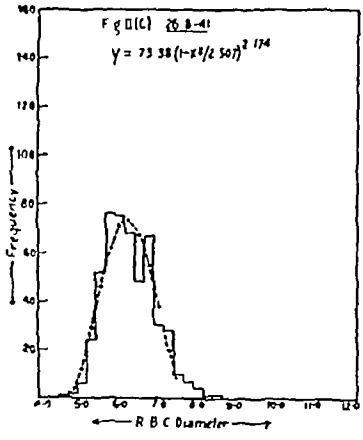


Fig II(c) 26 8 41
Frequency
R B C Diameter

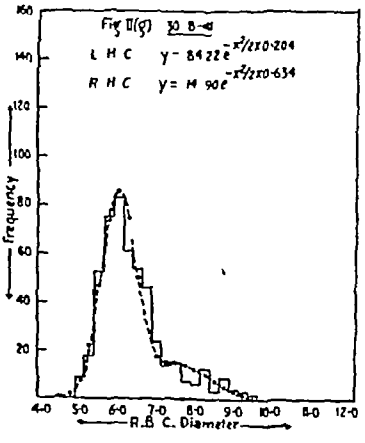


Fig II(g) 30 8 41
Frequency
R B C. Diameter

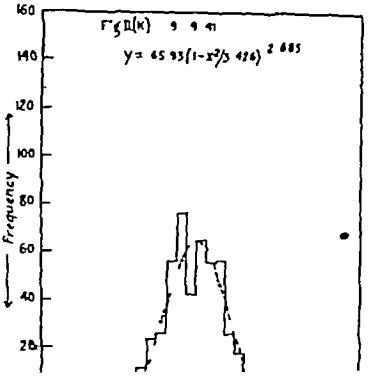
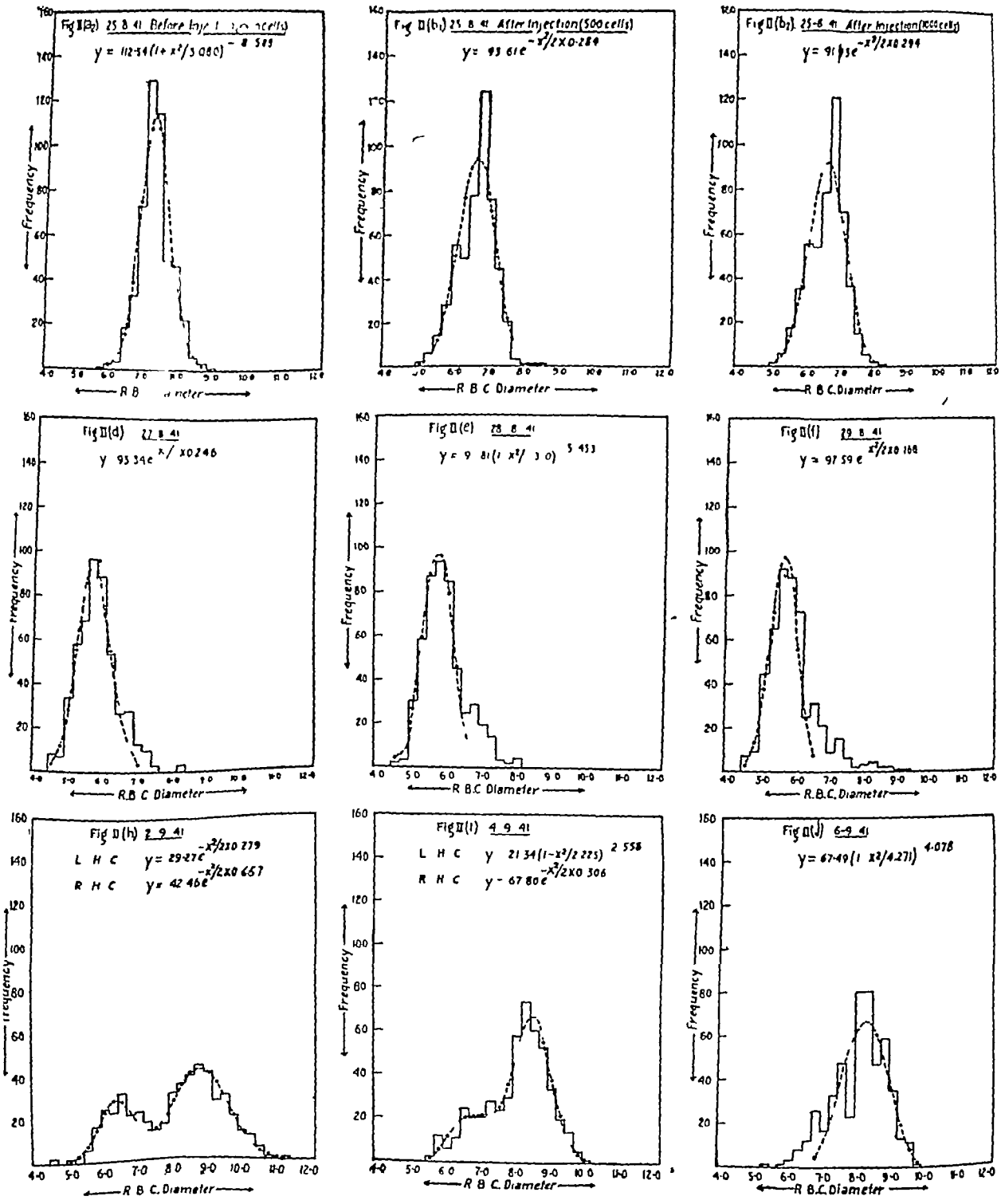


Fig II(k) 9 9 41
Frequency

FIGURE II.



The equations are given with means as originals

L H C = Left hand component
R. H C = Right-hand component.

Discussion —The general conclusion as to the effect of the single large dose of hæmolysin given intravenously is as follows There is production of an acute hæmolytic crisis starting almost immediately after the injection of hæmolysin The severe prostration, hæmoglobinæmia, hæmoglobinuria, and anæmia, indicate this condition That there is a sudden lysis of a considerable number of red cells is certain, though the blood count done immediately after may not show it, in all probability on account of certain degree of compensatory hæmoconcentration which is inevitable as a result of the general reaction caused by the hæmolysin The crisis passes away by the next day, the anæmia now becomes more apparent, and it is found that certain changes have taken place in the red cells The effect on the red cells appears to be that the cell diameter decreases and the thickness increases (spherocytosis), and there is an increase in fragility There is no recurrence of hæmolytic phenomena, but the spherocytic character of the cells remains, and the change in the cells seem to be progressive The cells not only progressively diminish in diameter but the mean cell volume also decreases It seems that the hæmolysin not only makes the cells spherocytic but causes a shrinkage of the cells day by day This fact (i e that the cells shrink in volume) is against the conception of Ponder that the red cell envelope is plastic but not elastic (Haden, *loc cit*)

The other explanation of the progressive fall of MCV and the MCD that suggests itself is that the cells of larger volume and diameter are destroyed earlier than the smaller cells

We have already seen that the effect of the hæmolysin on the existing cells of the monkey is to make them all spherocytic The cells that survive the acute hæmolytic crisis are smaller in volume as well as in diameter If this is because the cells with a larger MCV and MCD are removed first, it is difficult to explain why larger cells should be destroyed earlier than the smaller cells Eventually, all the spherocytic cells are slowly but surely lysed and after about three weeks no spherocytic cell remains

The new cells make their appearance from the 3rd day after the acute hæmolytic crisis, these cells are larger than the normal cells of the monkey, both in volume and diameter The appearance of these larger cells in the circulation is associated with a rise in the MCV, the MCD, and the reticulocyte curves From Plate I, fig 4, it will be seen that the new larger cells are actually reticulocytes

As the new cells are being formed, the proportion of spherocytes to new cells undergoes a striking but readily understandable change At first, 100 per cent of the cells are spherocytes, the new cells being entirely absent, then the proportion of new cells gradually increases, until at the end of about three weeks no more spherocytes are left and only the new cells are seen The spherocytes have a smaller diameter and the diameters form a symmetrical distribution curve The new cells are at first very irregular in size (and shape), but are on the whole large, that is, both the mean diameter and its standard deviation are large In the days following, the diameters of the new cells gradually assume a more uniform distribution and the mean diameter also comes down Ultimately, when only the new cells remain—the mean diameter and its standard deviation come down to within the normal range for the monkey

In this experiment the increase of fragility ran parallel to the increase in thickness of the cells, the more spherocytic the cell, the more fragile was it to hypotonic saline This bears out the experience of numerous observers regarding the mechanism of hæmolysis in acholuric jaundice (congenital hæmolytic icterus) or in the hypotonic hæmolysis of normal cells (For review of current work, *see* Haden, *loc cit*)

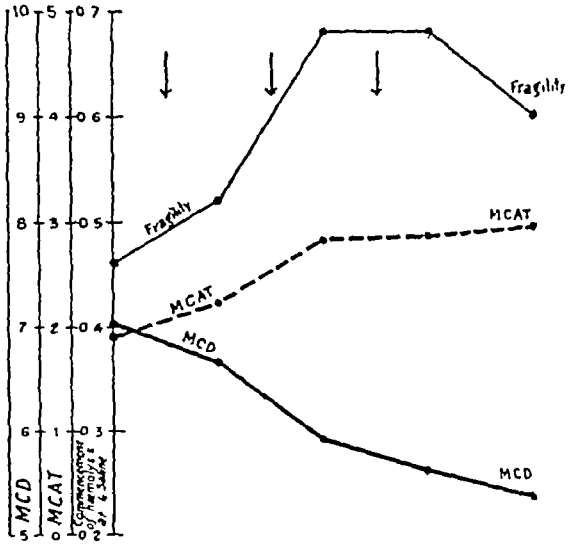
II Experiment using repeated large doses of hæmolysin

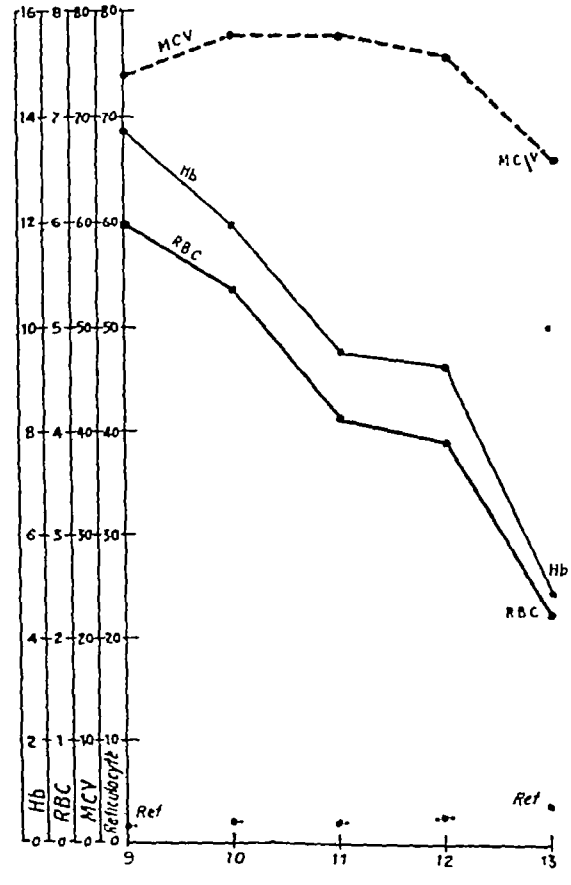
Here the monkey was given fairly large doses of hæmolysin repeatedly, the injections being given on consecutive days The first injection was a small dose (50 MHD), the three subsequent injections were given on the next 3 days, the dosage on each occasion was 400 MHD given intravenously undiluted The first injection produced only a slight reaction, though hæmoglobinuria was produced On the next 3 days, 400 MHD was given at about 11 a m and the blood was collected at 3 p m each day, all three injections were followed by severe reactions,

hæmoglobinæmia, and hæmoglobinuria The injections were discontinued after the third of this series as the monkey had severe prostration and the urine was markedly hæmoglobinuric and scanty On the next 2 days, there was slight hæmoglobinuria The urine showed the presence of albumin, hæmoglobin, and finely granular and hyaline casts The monkey was seriously ill, it developed severe diarrhœa, was markedly anæmic, and could hardly move Twenty-five c c of 25 per cent glucose was given intravenously Two days later the monkey died A post-mortem examination was made

GRAPH C

Monkey 3





Blood changes (*see* Table C and Graph C)—The hæmoglobin and red cell count dropped extremely rapidly during the few days the monkey survived Hæmoglobin came down from 13 75 g to 4 8125 g per cent and the red cells from 6 02 millions to 2 22 millions in 4 days Slight reticulocytosis was noticed on the 3rd day after the first injection of 400 MHD of hæmolysin The mean corpuscular volume decreased progressively in the course of the 4 days The mean cell diameter came down very rapidly (7 02μ to 5 32μ) and the thickness increased from 1 9μ to 2 9μ in these 4 days, and the fragility (commencement of hæmolysis) increased from 0 46 per cent to a maximum 0 64 per cent The van den Bergh test showed a marked rise of bilirubin content of plasma from 0 2 mg to 4 mg per cent

Result of post-mortem examination—It was found that there was fibrinous pleurisy of both lungs The heart showed no abnormality The gall-bladder was full of dark inspissated bile There was one small localized abscess in the left kidney—the smear of pus showed Gram-negative bacilli The urine in the bladder was quite clear and there was no hæmoglobin in it The bone-marrow in the shaft of the long bones was red in colour A smear from the marrow showed excess of erythropoietic cells

'HISTOLOGICAL NOTES'—BY N V BHADURI, M SC, M B

Kidney—This organ shows well-marked degenerative changes which are noticed in most of the tubules In a few of the glomeruli there is seen a slight serous exudate within Bowman's capsule The capillaries of the glomerulus are not congested and in many cases appear to have undergone shrinkage (? effect of the fixative) There is no leucocytic infiltration in the exudate within the capsule, nor in the capillaries Most of the convoluted tubules

PLATE I

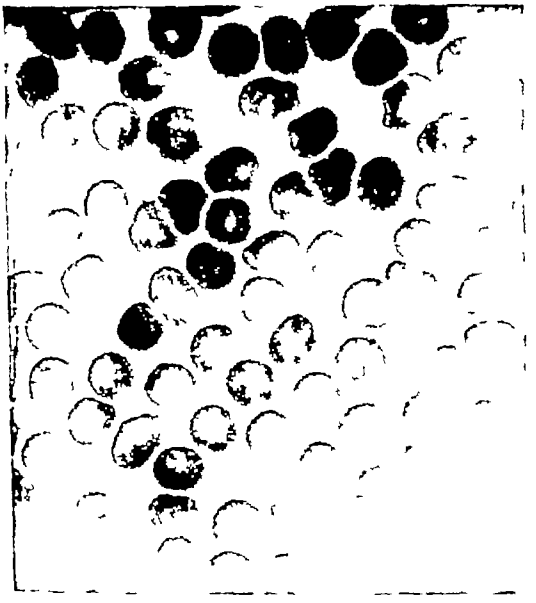


Fig 1 Monkey A. Before injection.
19th March, 1942

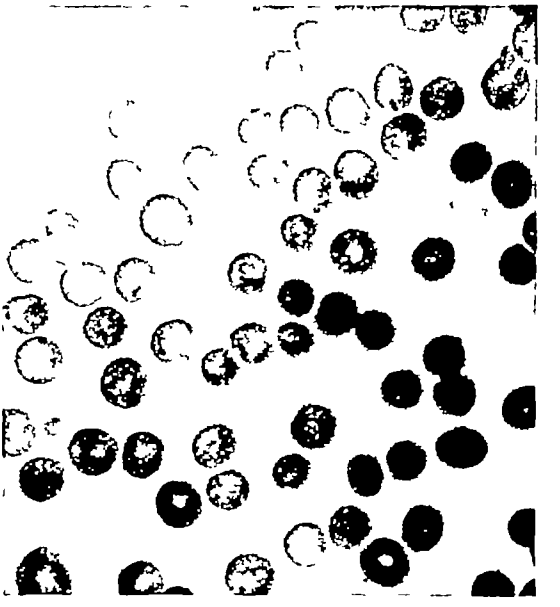


Fig 2 Two days after injection.
21st March, 1942

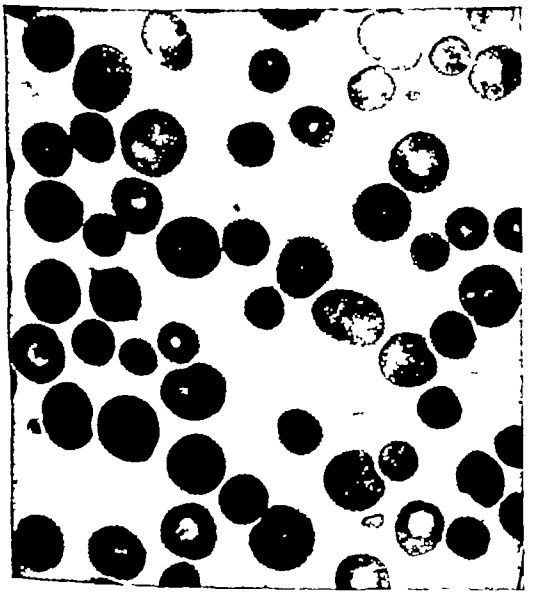


Fig 3 At the height of regeneration.
26th March, 1942

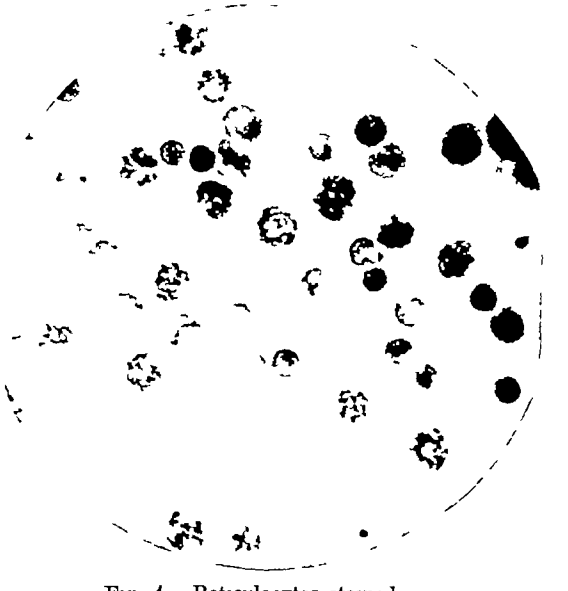


Fig 4 Reticulocytes stained

Plate II

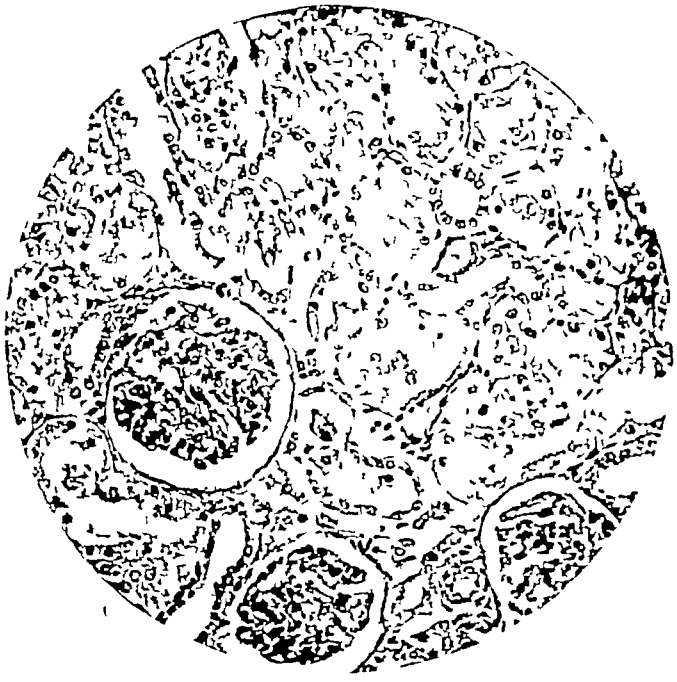


Fig 1 Section of the kidney showing changes in the glomeruli and convoluted tubules

Fig 2 Section of the kidney showing tubules filled with granular debris

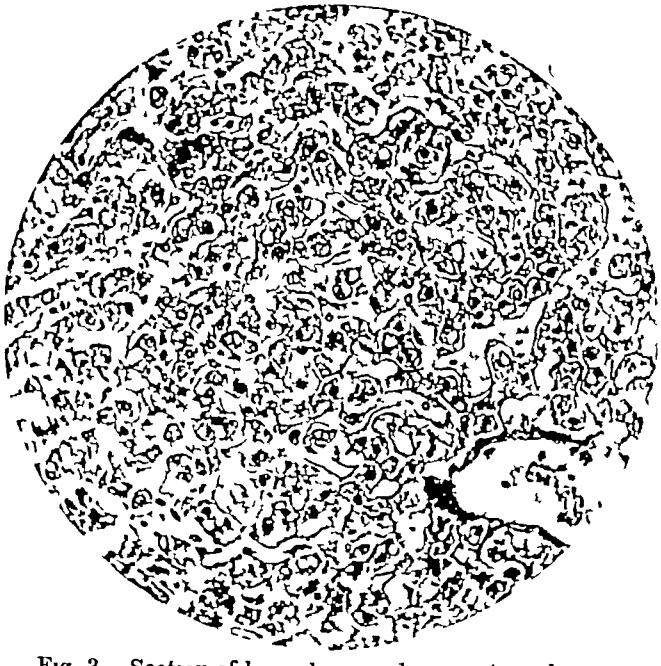


Fig 3 Section of liver showing degenerative changes in the parenchyma cells around the central vein of the lobule and dilated sinusoids

surrounding the glomeruli are filled with granular deposit, the lining cells are mostly undergoing granular changes and are broken down (see Plate II, figs 1 and 2) There is no inflammatory reaction in or around these tubules The ascending and descending tubules are also similarly affected and some of these in the deeper parts of the organ are seen to be choked The whole picture appears to be one of toxic degeneration without signs of inflammation and limited to the tubules

Liver—In this organ the cells mostly affected are those around the central veins of the lobule These cells are undergoing fatty changes and are often vacuolated and broken down The sinusoids between the strands of cells are dilated The cells in the peripheral zone of the lobule are not so much affected they show cloudy swelling and are stained more deeply than the central ones There is no congestion or leucocytic infiltration (see Plate II, fig 3)

Spleen—This organ is very congested Around the prominent Malpighian corpuscles the blood spaces are dilated and full of red blood cells These areas also show much deposit of pigment granules

Lungs—The only change noticed in this organ is that the vesicles are not fully expanded

Heart muscle—No changes are seen

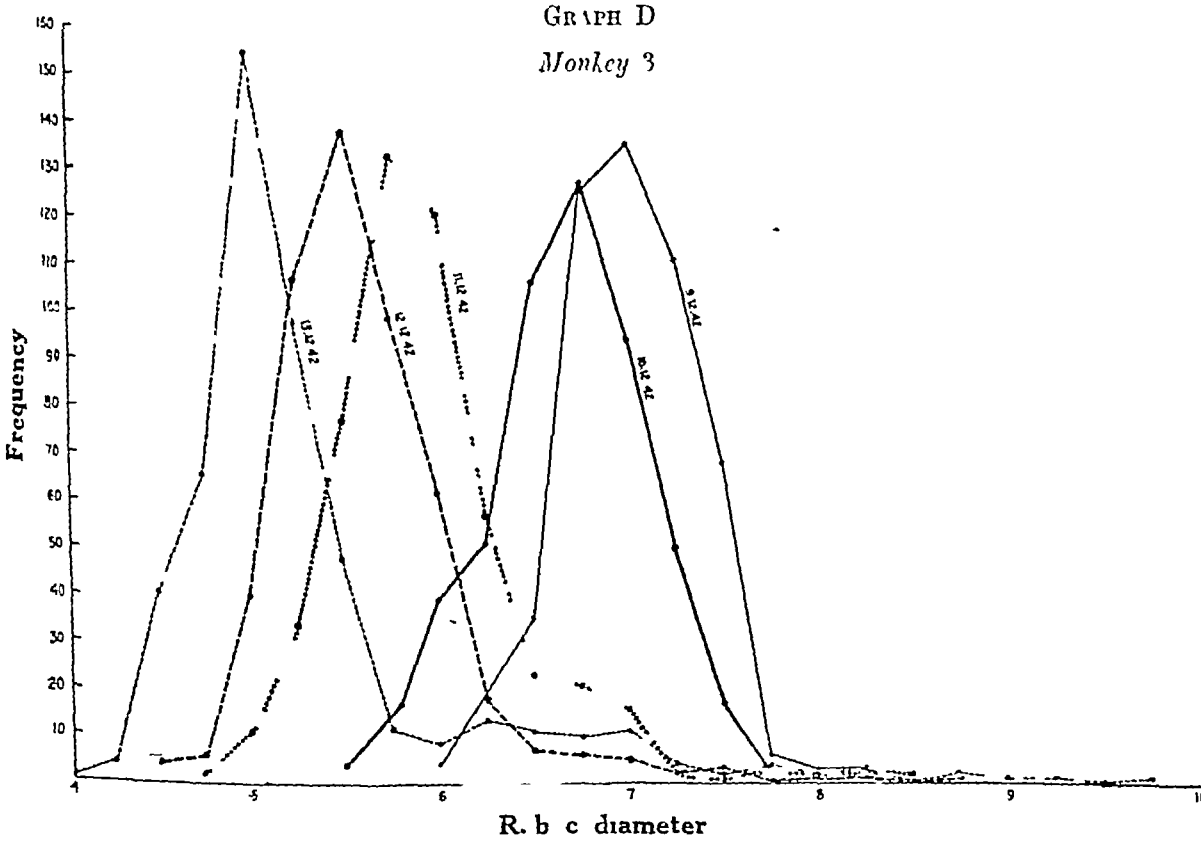
Bone-marrow—There appears to be some erythroblastic reaction

Discussion

The repeated doses of hæmolysin injected into the monkey produced a fulminating hæmolytic anæmia with repeated attacks of hæmoglobinuria, severe prostration, and a high

GRAPH D

Monkey 3



bilirubin content of blood, so severe was the anæmia produced that a terminal secondary infection supervened The degree of spherocytosis produced was remarkable and the cells became

extremely fragile, hæmolysis took place in 0 68 per cent sodium chloride The bone marrow was not inactive, no sharp rise of reticulocytes occurred though a slight degree of reticulocytosis was evident The Price-Jones' curves showed a marked day-to-day shift to the left (*see* Graph D) Evidence of slight new cell formation was present in the last three curves

Summary and conclusions

The Tables and Graphs show very explicitly what occurs to the circulating red cells when a hæmolytic serum is given intravenously

A massive hæmolysis occurs this is followed by hæmoglobinæmia and hæmoglobinuria The remaining blood cells are now markedly spherocytic These cells show increasing susceptibility to hypotonic saline solutions, but apparently not to any great extent to normal hæmolytic processes, since some of them survive for nearly three weeks (against the normal life of four weeks of the monkey's red cells)

As these cells disappear from the circulation they are replaced by larger cells which are not spherocytic and withstand hypotonic saline normally, these cells can be shown to be reticulocytes The reticulocytes as they mature become reduced in diameter, so that eventually the Price-Jones' curve is the same as before any hæmolytic serum was given

After the action of the hæmolysin the mean corpuscular volume (MCV) of the red cells is also reduced Thus, both the red cell diameters and volume are reduced, the explanation of this is not clear, and this point is discussed

It would thus appear that though the life of the spherocyte may possibly be shorter than that of the normal cell, it cannot be considered that the assumption of the spherocytic shape is an *immediate* prelude to normal hæmolysis It suggests rather that, though the assumption of the spherocytic shape may lead to intravascular disintegration of the red cell, if the effect falls short of this, it does not materially shorten the life of the red cell A similar state of affairs exists in congenital hæmolytic icterus, where removal of the spleen stops the increased rate of destruction of red cells, but does not markedly affect the spherocytosis

The observation indicates that the mechanism of *excess* hæmolysis resulting from injection of a hæmolytic serum may be similar to the mechanism of excessive blood destruction in congenital hæmolytic anæmia (and possibly to that in blackwater fever), but is quite different from that of physiological hæmolysis

Acknowledgment

The inquiry was conducted at the School of Tropical Medicine, Calcutta, with a grant from the Indian Research Fund Association, for which our thanks are due to the Association

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TABLE A

Monkey A1

Date	Hb, g per cent	RBC, millions per c mm	Ret., per cent of RBC	CV, per cent	MCV, cu μ	MCH, $\gamma\gamma$	MCHC, per cent	MCAT, μ	Haemolysis	
									Comm	Compl
									Per cent NaCl soln	
19-3-42	11 6875	5 53	1 6	39	70 52	21 13	29 96	1 74	0 38	0 24
19-3-42	11 6875	5 56	1 3	39	70 14	21 02	29 96	1 98	0 52	0 26
20-3-42	11 55	5 23		32	61 18	22 08	36 09	2 003	0 52 +	0 34
									0 50 ±	
21-3-42	9 075	4 72	3·0	26	55·08	19 22	34 90	2 11	0 50 +	0 30
									0 48 ±	
23-3-42	7 5625	3 43	0 3	23	67 05	22 04	32 88	2 33	0 48	0 28
24-3-42	7 8375	3 10	41 6	23 5	75 80	25 28	33 34	2 13	0 46 +	0 28
									0 44 ±	
25-3-42	8 25	3 59	30 0	26 5	73 81	22 98	31 13	1 52	0 46 +	0 28
									0 44 ±	
26-3-42	8 25	3 26	30 4	27 0	82 82	25 30	30 55	1 93	0 44 +	0 25
									0 42 +	
									0 40 ±	
27-3-42			29 2							
28-3-42			19·0							
30-3-42	8·9375	4 10	9 0	30 5	74 39	21 79	29 30	1 59	0 36	0 22
2-4-42	9 215	4 60	8 2	33 5	72 82	20 02	27 5	1 54	0 38 + ?	0 24
									0 36	
7-4-42	10 3125	5 170	2 6	35	67 69	19 94	29 46	1 60	0 38	0 22

TABLE B

Monkey 7

Date	Hb, g per cent	RBC, millions per c mm	Ret., per cent of RBC	CV, per cent	MCV, cu μ	MCH, $\gamma\gamma$	MCHC, per cent	MCAT, μ	Haemolysis	
									Comm	Compl.
									Per cent NaCl soln	
25-8-41	14·025	5 99	0 8	46 5	77 62	23 41	30 16	1 78	0 46	0 36
25-8-41	13·06	5 33		43 5	81 61	24 50	30 02	2 39	0 64	0 38
26-8-41	14 3	5 86	1 6	44	75 08	24 40	32 5	2 451	0 64	0 42
27-8-41	12 1	4 93	2 4	33	66 93	24 54	36 66	2 48	0 60	0 38
28-8-41	9 9	3 70	3 8	27	72 97	26 75	36 6	2 76	0 56	0 38
29-8-41	8 8	3 63	4 2	23 5	64 73	24 24	37 4	2 406	0 56	0 38
30-8-41	7 56	3 06	10 1	22	71 89	24 71	34 375	2 25	0 56 + ?	0 32
									0 52 + ?	
									0 50 ± ?	
2-9-41	8 8	3 02	27	31 25	103 48	29 13	28 16	2 05	0 48	0 22
									0 46	
4-9-41	8 8	3 64	25 8	34 6	95 05	24 17	25 43	1 90	0 48	0 26
6-9-41	9 9	3 64	8 4	34 5	94 78	27 19	28 69	1 87	0 44	0 26
9-9-41	11 275	4 48	8 4	39 75	88 72	25 10	29 48	1 86	0 42	0 24
12-9-41	12 1	4 72	4 2	43	91 10	25 6	28 1	1 98	0 40	0 22
									0 38	
16-10-41	15 125	6 13	0 5	48 5	79 1	24 67	31 18	0 44	0 44	0 32

TABLE C

Monkey 3 (repeated injections of hæmolysin)

Date	Hb, g per cent	RBC, millions per c mm	Ret., per cent of RBC	CV per cent	MCV cu μ	MCH, $\gamma\gamma$	MCHC, per cent	MCD, μ	Standard deviation (σ)	Coefficient of variation (v)	MCAT, μ	Hæmolysis	
												Comm	Compl
												Per cent NaCl soln	
9-12-41	13 75	6 02	1 4	44 5	73 92	22 84	30 89	7 0255	0 3475	4 94	1 909	0 46	0 30
10-12-41	11 9625	5 35	2 0	41 5	77 57	22 35	28 82	6 674	0 4417	6 6	2 220	0 52	0 34
11-12-41	9 4875	4 11	2 0	31 75	77 25	23 08	29 88	5 908	0 4587	7 7	2 821	0 68	0 44
12-12-41	9 2125	3 90	2 6	27 5	70 51	23 62	33 50	5 608	0 490	8 7	2 856	0 68	0 44
13-12-41	4 8125	2 22	3 6	14 5	65 31	21 67	33 18	5 3205	0 7975	14 9	2 943	0 60	0 44

'STATISTICAL ANALYSIS'—BY C CHANDRA SEKAR, M SC, PH D (Lond)

Introduction—A cursory glance at the distributions of the red blood cell diameters on different days, before and after the injection, given in Tables I-*a* and I-*b*, will show clearly that considerable time has to elapse before the distribution curve attains equilibrium and regains the location and shape it had before the monkey was injected In the disturbed condition two features stand out (1) that the new cells formed a few days after the injection are initially of much larger diameters than are common in monkeys, and (2) that the cells existing before injection and formed subsequently by a gradual change whether accompanied or not by differential disintegration finally result in a normal distribution of the red blood cell diameters The object of this note is to describe in quantitative terms the distribution curves in the different stages with a view to a better understanding of the changes in the red cell diameters following the injection

The statistical problem

Figures I and II give in the form of histograms the distribution curves of the red cell diameters on different days for monkeys A and 7, respectively From the 4th day after the injection for monkey A and the 3rd day after the injection for monkey 7, the histograms definitely show the distribution curves as bi-modal and this feature persists markedly till the 14th day for monkey A and the 10th day for monkey 7 Since of these two components it is not unlikely that one refers to the cells which were present before the injection and the other to those formed subsequently, the statistical problem is to resolve wherever possible the distribution curves into its two components

The problem of the decomposition of a frequency distribution into two or more components is very old but a number of theoretical and practical difficulties have stood in the way of a satisfactory method of solution, of wide generality, from being evolved Karl Pearson (1894) has given a method of decomposing asymmetrical and symmetrical distributions into two normal curves of error, and as a first step this method necessitates the determination of the roots of a ninth degree equation Dr Rasch (in Mogensen, 1938) suggests a graphical method of decomposing a Price-Jones' curve into one main component represented by a normal curve of error and two minor components one on either side of this normal curve Though this method has the advantage of offering an easy practical solution it fails to give the confidence of a purely algebraical treatment

Both the methods referred to above make assumptions concerning one or more of the components But in discussing the distribution curves of the red cell diameters for monkeys

it is difficult to make *a priori* any such assumptions It is indeed not known if, as in human beings even normally this curve will take the form of a normal curve of error So some indication of the type of curves which would represent the shape of the two components is first necessary and in case both happen to be normal curves of error the method suggested by Karl Pearson (*loc cit*) may be used to describe them It would certainly be worth while devising easier methods of describing quantitatively the two components, whether they happen to be normal curves of error or not

Method used in describing the distribution curves

Pearson found that even if a distribution curve follows a smooth symmetrical or asymmetrical distribution the data represented by it need not be homogeneous and he was successful in dividing up such smooth curves into two components Such decompositions of even smooth frequency distributions of the red cell diameters may be of theoretical interest, but for the problem under investigation it appeared good enough to assume that the cells with diameters represented by smooth curves preferably of the Pearsonian type (Elderton, 1938), were homogeneous Therefore except when a single smooth curve failed to fit the data or when the bi modal nature of the distribution was obvious, there was no need to resolve the data into two components

The process of decomposition of curves may be divided into two stages —

(1) Specification of the type of curves by which the components are to be represented

(2) Estimation of the relative frequencies in the two components and of the parameters for the different curves

To get an idea of the types of curves representing the components purely by algebraic methods is extremely difficult and so the following procedure was adopted By drawing smooth curves over the histograms in Figs I and II an attempt was made to find out if the distributions were uni-modal or bi-modal If bi modal these smooth curves were used in dividing up the cell diameters into two distributions, each one referring to one of the components For each of these distributions, except when the frequency was small, a smooth curve was fitted by Pearson's method of moments Whenever two smooth curves were drawn the fit of both the curves to the original data was tested simultaneously by applying the χ^2 test The results of applying the χ^2 test are given in Tables D and E for monkeys A and 7 respectively and in general the curves give a good fit To facilitate a visual impression of the degree to which the smooth curves fit the data, these curves are drawn over the histograms in Figs I and II Since the fit is good enough it is reasonable to assume that the type of smooth curves obtained by the above procedure are adequate to represent the two components

The next step was to estimate the relative frequencies of the components and the parameters of the smooth curves, starting only with the simple assumption that the types of these smooth curves are as indicated by the first analysis In this communication the investigation has been carried only to the first stage, viz the specification of the type of curves describing the components In the course of this work, however, results are obtained which give an approximate solution to the main problem, viz an adequate description of the distribution curves, and it has been considered useful to summarize the tentative conclusions drawn from these results It was expected that at a later date it would be possible to offer a more rigorous treatment of the data

The relative frequencies, the mean, standard deviation and Pearsonian β_1 and β_2 (except when the frequencies in a component are few), of the components when the distribution was resolved and of the total distribution when it was not necessary to decompose it, are given in Tables II-*a* and II-*b* for monkeys A and 7 respectively

Description of the distribution curves—Monkey A

Before injection, 19-3-42 —The 500 cell diameters give a mean of 7 161μ, a standard deviation of 0 3935μ and Pearsonian β_1 and β_2 of 0 0102 and 3 5532 respectively Since β_1 is small and β_2 greater than that of the normal curve of error it is to be expected that a symmetrical curve with a greater cluster of values round the mean than in the normal curve of error with the same standard deviation represents the actual distribution Such a curve, however, did not give a very good fit In order to get a better idea of the true distribution another 500 cell diameters were measured and a symmetrical curve of the type first fitted was again tried The fit did not improve very much From the values of β_1 and β_2 for the 1,000 cell diameters it seems that a slightly asymmetrical curve with a larger number of values above the mean may give a better fit and such a curve is drawn over the histogram in Fig I (*a*) For practical purposes, however it is reasonable to take the distribution as symmetrical but having a greater peak than the normal curve of error 95 per cent of the cell diameters lie between 5 875μ and 8 375μ

After injection, 19-3-42 —After injection the mean of 500 cell diameters drops down considerably to 6 705μ The standard deviation is much the same as before the injection The value of β_1 the index of asymmetry increases due to a slightly longer tail for values below the mean but the distribution curve cannot be considered asymmetrical The shape of the new curve indicates that cell diameters become more uniform after the injection and a larger proportion cluster round the mean Cells with much smaller diameter than was met with before injection now appear and about 0 7 per cent cells are below 5 675μ The standard deviation remaining unchanged and β_2 increasing does not suggest that all cells had changed their diameters proportionately

1st day after injection, 20-3-42 —The 500 cells give a β_1 equal to 0 1776 showing that the distribution curve is very asymmetrical It appears from the histograms that if only a few cells with higher diameters be removed the remaining cells would prove symmetrical Seventeen values from the extreme right-hand tail of the curve were separated by inspection and a smooth symmetrical curve was fitted to the other 483 cell diameters The mean of this component is 6 201μ and the standard deviation 0 424μ This curve is much flatter than the ones after and before the injection The mean of the 17 cells is 7 325μ and standard deviation 0 352μ It is indeed difficult to say if new cells had appeared and were responsible for the 17 values which were considered to be outside the main component But the increase in reticulocytes seem to point towards this possibility

A large number of cells with smaller diameters are now met with, about 8 8 per cent cells having diameters smaller than 5 675μ

2nd day —The histogram points to an abruptness for the higher cell diameters By inspection 472 cells were taken as forming the main component This component has a mean 5 671μ, is symmetrical but is more peaky than the curve on the previous day 21 8 per cent cells have diameters below 5 375μ Though this symmetrical curve gives a very good fit to the 472 cell diameters, yet it is not impossible that such a fit could be obtained with the help of an asymmetrical curve fitted to all the 500 observations

The mean of 28 cells forming the minor component is 7 223μ and the standard deviation 0 5400μ

4th day —On this day the histograms clearly point to two components, the major one containing 406 cells The mean diameter of these cells is 5 509μ and the standard deviation 0 4261μ The minor component of 94 cells has a very high standard deviation of 0 9713μ and the cell diameters are also large Whereas no cell of diameter greater than 8 5μ was noticed on the 2nd day after the injection, the mean of these 94 cells is 8 39μ and 4 5 per cent of the total number of cells have diameters greater than 9 125μ Evidently, this minor component is due to new cells

5th, 6th and 7th days —For these 3 days the histograms show up clearly the two components The percentage of cells included in the component attributable to the new cells

is 36 0 on the 5th day 50 2 on the 6th day and 58 1 on the 7th day This increase is consistent with the gradual increase noticed earlier

The means of the components on the left hand side on these 3 days are 5 738μ 6 024μ and 5 894μ The mean on the 5th day is higher than the corresponding value on the 4th day and this difference is significant This upward trend is maintained also on the 6th day The increase in mean diameter is not in conformity with the decrease noticed earlier The main reason for this is that on the 4th day cells of very small diameters were found and in fact 13 7 per cent of total cells had a diameter below 5 125μ But on the 5th and 6th days such small diameters became rarer with the result that on the 5th day 1 3 per cent and on the 6th day 0 75 per cent cells have diameters below 5 125μ On the 7th day the mean is significantly smaller than on the 6th day This is not due to the presence of cells with very small diameters though the cells become smaller and more uniform The slight tendency for more values above the mean present on the 5th and 6th days also disappears making the curve very symmetrical

As regards the component on the right hand side the tendency is for the values to cluster more near the mean and that is why the standard deviation decreases and β_2 shows an upward trend The new cells with larger diameters are formed in large numbers on the 5th and 6th days as shown both by the large proportion of cells with higher diameters and the higher reticulocyte count Whereas, on the 4th day only 1 5 per cent cells had diameters greater than 9 125μ this percentage was 9 1 on the 5th day and 23 6 on the 6th day It is not surprising that the mean on the 6th day is as high as 9 070μ On the 7th day cells of diameters greater than 10 0 become rare suggesting that the new cells gradually reduce in size

11th, 14th and 19th days—The left hand component becomes smaller and smaller the proportion of frequencies in this component being 8 8 per cent on the 11th day, 7 6 per cent on the 14th day and 3 0 per cent on the 19th day The cell diameters in this component range from 5 25μ to 6 75μ up to the 14th day and from 5 50μ to 6 25μ on the 14th day The means for these components are relatively constant on these days being in the neighbourhood of 5 95μ

After the 11th day the reticulocyte count goes down and the component on the right-hand side shifts towards the left Whereas 11 1 per cent of the cells were above 8 625μ on the 11th day, this percentage reduces to 1 7 on the 14th day and 0 72 per cent on the 19th day The percentage of cells of this component having values below 6 625μ is 2 3 on the 11th day, 1 8 on the 14th day, and 6 1 on the 19th day Towards the 19th day fewer values of larger diameters and more values of smaller diameters are met with and this explains the mean falling down to 7 400μ on the 19th day from 7 888μ on the 11th day The standard deviation goes down and more cells cluster round the mean On the 19th day the mean and standard deviation are greater than before the injection but the tendency is for the position and shape of this component to regain the form of the distribution curve before the injection

Description of the distribution curves—Monkey 7

Date of observation, 25 8-41 (*before injection*)—The first 500 cells measured give a mean of 7 451μ and standard deviation 0 4360μ The value of $\beta_1 = 0\ 0330$ does not suggest asymmetry but the high value of β_2, viz 3 5218, indicates that the distribution curve is more peaky than the normal curve A symmetrical Pearsonian curve does not give a good fit Another 500 cells were measured to get a better idea of the actual distribution and the 1,000 cells together indicate the same type of curve as the one fitted previously The fit of such a curve is not however satisfactory The reason for the bad fit is that in both cases the observed frequency is greater for cells with diameters 7 25μ and less for cells with diameters 7 75μ than that given by the curve Whatever may be the true distribution, it is expected that the mode will be near the estimated mean of 1,000 cells, viz 7 3855μ

After injection—The mean drops down considerably to 6 591μ for the 500 cells The standard deviation shows a slight increase and the values of β_1 and β_2 indicate that a normal curve of error would adequately describe the distribution curve Such a curve, however, gives

a bad fit due to an excess of observations at 6 75μ and a defect at 6 25μ Another 500 cells were measured, in view of the bad fit but the 1,000 cells gave exactly the same results as the first lot of 500

1st day —The major component has 482 cells with a mean of 6 1883μ and standard deviation 0 5842μ The distribution curve also becomes flatter with a slight increase in the proportion of cells with small diameters The minor component has a mean of 7 8055μ.

2nd, 3rd and 4th days —The proportionate frequencies in the major component gradually decrease being 92 8 per cent on the 2nd day, 84 8 per cent on the 3rd day and 80 2 per cent on the 4th day The mean diameter for these components also gradually decrease and come down to 5 567μ on the 4th day

On the 2nd day β_2 increases and the cells cluster more round the mean This tendency for the evening up of the cell diameters is well accentuated till the 4th day

The minor components have erratic distributions and on the 4th day cells with diameters as large as 9 0μ are noticed

5th and 8th days —The left-hand component consists of 381 cells on the 5th day and 155 cells on the 8th day The mean diameters of these components are respectively 5 9921μ and 6 350μ These increases over the mean diameter on the 4th day are significant and are due to the reduction of cells of small diameters Whereas 11 3 per cent of the total number of cells were below 5 125μ on the 4th day, only 2 1 per cent were below the value on the 5th day and only 2 6 per cent were below 5 625μ on the 8th day

More and more new cells with large diameters appear and the production of new cells appear to attain a maximum about the 7th or 8th day

10th, 12th, 15th and 18th days —The left-hand component decreases in size 24 8 per cent of all cells being included in it on the 10th day, 10 0 per cent on the 12th day, 6 6 per cent on the 15th day and 2 6 per cent on the 18th day The mean diameter of this component decreases continuously, not due to smaller cells making their appearance but due to the larger cells getting reduced This is shown by the standard deviation decreasing continuously

The component due to the new cells has a mean diameter of 8 431μ, a standard deviation of 0 5531μ and a β_2 of 2 8520 on the 10th day This decrease in standard deviation and increase in β_2 compared with their corresponding values on the 8th day points to a greater clustering of values round the mean The decrease in mean is due to the absence of cells with very large diameters for as compared with 5 9 per cent of the total number of cells above 9 875μ on the 8th day the corresponding percentage on the 10th day is only 0 3 The component on the right-hand side shifts to the left marked by a growing absence of cells with large diameters From the 10th to the 15th day the cells spread out with the result that on the 12th day 4 3 per cent and on the 15th day 12 7 of this component have cell diameters below 7 125μ On the 18th day also the same tendency for a lateral shift to the left accompanied by an increase of the cells of smaller diameters and a decrease of cells with larger diameters can be noticed But now β_2 increases while the standard deviation slightly decreases showing that the shape of the curve is tending to the peaked form which the distribution curve manifested before the monkey was given the injection

Summary of changes in RBC diameters following the injection —The distribution curves before injection are fairly symmetrical but have a greater peak than the normal curve of error The mean diameters in the two monkeys are significantly different but the standard deviations are of the same order

Immediately after the injection the mean cell diameters fall considerably and in the case of monkey A there is a definite tendency for more cells to cluster round the mean Cells of diameters much smaller than were encountered before the injection make their appearance

On the 1st day after the injection the mean cell diameter falls much further but the distribution curves become much flatter and there is less of a cluster around the mean

Up to the 4th day the sizes of the main component decrease slowly and the mean cell diameters of these components continue to go down These are brought out clearly

in Figs III and IV It should be observed that cells of very small diameters are now noticeable

FIGURE III

Graph showing the proportion of old cells on various days after the injection

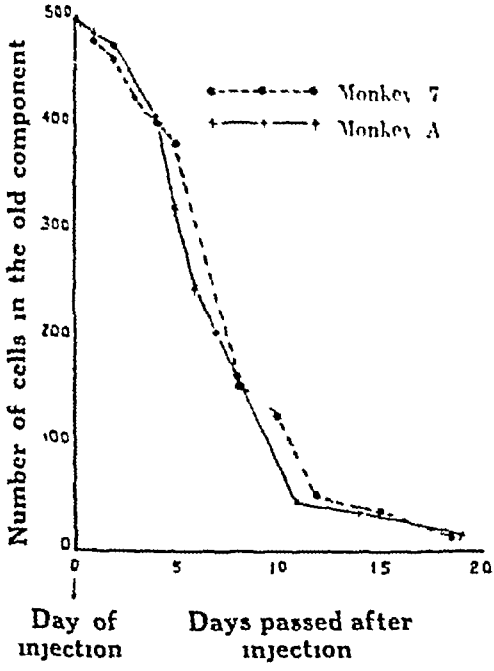
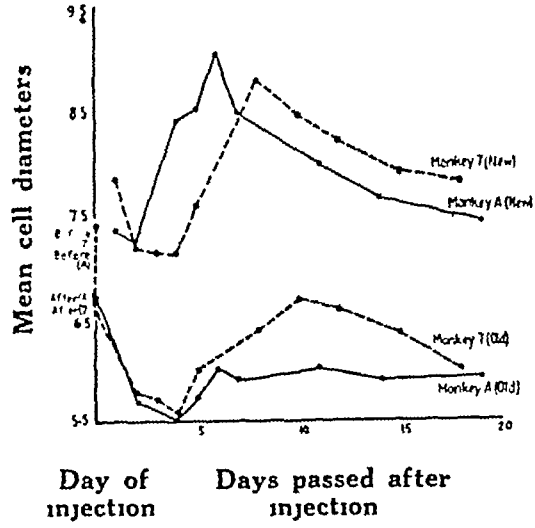


FIGURE IV

Graph showing the mean cell diameters of the old and new components



On the 5th day the mean cell diameter of the component of the old cells increases slightly This is not due to any general increase in the sizes of the cells , on the contrary the tendency is for the cell diameters to decrease but the effect of this is offset by the elimination of cells of very small diameter and cells below 5 000μ are rare Increase of mean cell diameter due to this cause is clearly noticeable in monkey 7 even on the 8th day

From the 5th to the 8th day new cells are being formed at a fast rate These cells are initially large and vary widely in size but after the 8th day the component of new cells shifts bodily to the left and the mean cell diameter gradually becomes smaller Also the standard deviations decrease signifying that the cells are becoming more homogeneous in size

After about the 18th day the old cells have nearly disappeared their rate of the disintegration being very slow after the 10th day The mean cells diameter of the new cells even so many days after the injection is higher than its value before the injection but the tendency is for the distribution to regain its original form and location

Acknowledgment

The heavy computational work involved in this investigation was willingly undertaken by Messrs K B Das Gupta and H P Chatterjee of the Section of Vital Statistics and Epidemiology of the All-India Institute of Hygiene and Public Health, Calcutta, and it is with pleasure that their assistance is acknowledged

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TABLE D

Showing the frequency distributions and the goodness of fit of curves worked out to represent the distributions of RBC diameters on various days for monkey A

	(a_1)	(a_2)	(b)	(c)	(d)	(e)
Date of observation —	19–3–42 (before injection)	19–3–42 (before injection)	19–3–42 (after injection)	20–3–42 (1st day after injection)	21–3–42 (2nd day after injection)	23–3–42 (4th day after injection)
Cell diameter	Frequency (complete distribution)	Frequency (complete distribution)	Frequency (complete distribution)	Frequency (major component)	Frequency (major component)	Frequency (complete distribution)
4 00						0 9
4 25						1 9
4 50					3 4	5 9
4 75			0 2		9 8	17 2
5 00			0 3	1 7	29 5	42 8
5 25			0 8	10 0	66 1	80 1
5 50	0 2		2 3	32 3	105 6	101 4
5 75	0 6	2 0	7 0	67 1	113 7	82 5
6 00	2 4	5 3	21 4	97 7	81 9	45 2
6 25	9 0	18 8	58 0	106 8	41 3	19 3
6 50	29 0	59 6	114 8	87 4	15 1	8 3
6 75	70 9	147 6	137 8	52 6	4 4	5 2
7 00	119 2	248 7	94 3	21 6	1 4	5 0
7 25	127 2	256 9	41 5	5 9		5 9
7 50	85 5	162 0	14 1			6 6

7 75	38 5	68 1	1 7			7 5
8 00	12 8	22 1	1 6			8 0
8 25	3 5	6 3	0 6			8 4
8 50	0 0	2 6	0 2			8 4
8 75	0 3		0 1			8 1
9 00						7 6
9 25						6 8
9 50						5 7
9 75						4 6
10 00						3 3
10 25						2 0
10 50						0 9
10 75						0 2
TOTAL	500 0	1000 0	500 0	183 1	172 2	199 7
χ^2 value	15 165	17 432	13 604	9 923	6 742	47 271
REMARKS	Probability of χ^2 exceeding 15 165 is between 0 02 and 0 05	Probability of χ^2 exceeding 17 432 lies between 0·02 and 0 05	Probability of χ^2 exceeding 13 604 is about 0 1	Probability of χ^2 exceeding 9 923 is about 0 20	Probability of χ^2 exceeding 6 742 is greater than 0 5	Probability of χ^2 exceeding 47 271 is very small, the bad fit is due to the distribution of the 94 cell diameters belonging to the minor component being very erratic

TABLE D—*concld*

Showing the frequency distributions and the goodness of fit of curves worked out to represent the distributions of RBC diameters on various days for monkey A

	(f)	(g)	(h)	(i)	(j)	(k)
Date of observation →	24–3–42 (5th day after injection)	25–3–42 (6th day after injection)	26–3–42 (7th day after injection)	30–3–42 (11th day after injection)	2–4–42 (14th day after injection)	7–4–42 (19th day after injection)
Cell diameter	Frequency (complete distribution)	Frequency (complete distribution)	Frequency (complete distribution)	Frequency (major component)	Frequency (major component)	Frequency (major component)
4 00						
4 25						
4 50	1 8					
4 75	4 8	0 9	6 8			
5 00	14 9	2 8				
5 25	36 8	9 5	15 5			
5 50	65 7	25 6	31 8			
5 75	79 5	48 6	46 1			
6 00	63 2	62 6	47 3			2 7
6 25	35 6	52 8	34 5	3 8	1 1	7 3
6 50	15 4	29 8	18 1	6 6	7 1	19 8
6 75	7 7	12 0	7 6	14 4	22 3	41 9
7 00	6 8	4 3	4 9	27 0	45 3	69 9
7 25	8 7	2 5	7 7	43 2	69 2	91 1
7 50	11 1	3 7	14 6	59 4	84 7	93 5

7 75	14 2	6 8	24 6	69 7	84 9	75 1
8 00	16 4	11 8	35 4	70 5	69 8	47 4
8 25	18 2	18 3	43 6	60 7	46 0	23 5
8 50	18 8	25 2	46 0	45 1	22 9	9 1
8 75	18 1	30 9	41 5	28 5	7 5	3 6
9 00	16 3	33 7	31 9	15 5	1 2	
9 25	14 1	33 0	21 0	7 2		
9 50	11 1	28 7	11 9	2 9		
9 75	8 2	22 2	5 7	1 4		
10 00	5 5	15 3	2 4			
10 25	3 4	9 4	0 8			
10 50	1 9	5 2	0 2			
10 75	0 9	2 5	0 1			
11 00	0 6	1 1				
11 25		0 6				
Total	499 7	499 8	500 0	455 9	462 0	484 9
χ^2 value	26 15	19 253	28 43	10 020	7 729	28 962
Marks	Probability of χ^2 exceeding 26 15 by chance is greater than 0 1	Probability of χ^2 exceeding 19 253 is greater than 0 3	Probability of χ^2 exceeding 28 43 lies between 0 01 and 0 02	Probability of χ^2 exceeding 10 020 is greater than 0 5	Probability of χ^2 exceeding 7 729 lies between 0 3 and 0 5	Probability of χ^2 exceeding 28 962 is very small but the high value of χ^2 is due solely to a great deficiency of observations at 7 75μ which is made up by an excess at 7 50μ and 8 00μ

TABLE E

Showing the frequency distributions and the goodness of fit of curves worked out to represent the distributions of RBC diameters on various days for monkey 7

	(a_1)	(a_2)	(b_1)	(b_2)	(c)	(d)	(e)
Date of observation —	25–8–41 (before injection)	25–8–41 (before injection)	25–8–41 (after injection)	25–8–41 (after injection)	26–8–41 (1st day after injection)	27–8–41 (2nd day after injection)	28–8–41 (3rd day after injection)
Cell diameter	Frequency (complete distribution)	Frequency (complete distribution)	Frequency (complete distribution)	Frequency (complete distribution)	Frequency (major component)	Frequency (major component)	Frequency (major component)
4 00							
4 25						5 3	0 7
4 50					2 2	12 4	7 1
4 75				4 3	12 6	29 7	27 0
5 00			1 5	10 7	28 8	55 6	58 9
5 25			4'1	28 7	46 5	81 1	87 4
5 50			11 9	62 2	61 4	92 3	96 2
5 75	0 2	1 6	27 3	109 5	70 9	82 1	78 5
6 00	1 1	3 7	50 7	156 5	72 9	56 6	46 5
6 25	3 3	11 7	76 0	181 5	67 0	30 6	17 9

6 50	10 5	33 2	91 4	170 8	54 7	12 8	3 6
6·75	20 4	70 5	88 7	130 2	37 0	5 5	
7·00	64 9	149 7	69 5	81 0	20 3		
7 25	104 7	211·0	43 6	40 7	6 8		
7 50	117 7	214 3	22 2	16 6			
7 75	90·0	155 0	9 1	5 5			
8 00	48 9	84 7	3·0	1 5			
8 25	19 9	36 1	0 8	0 4			
8 50	6·7	12 9	0 2				
8 75	2 8	4 1					
9·00		1 7					
TOTAL	500 1	1000 1	500 0	1000 1	482 0	404 0	123 8
χ^2 value	31 965	43 149	32 707	63 401	22 435	12 851	6 477
REMARKS	Probability of χ^2 exceeding 31 965 is very small, but the high value is almost entirely due to a deficit of values at 7·75μ and an excess at 7 25μ	Probability of χ^2 exceeding 43 149 is small, but again the high value of χ^2 is almost entirely due to a deficit of values at 7 75μ and an excess at 7 25μ	Value of χ^2 is high due to an excess of observed values at 6 75μ and a deficit at 6 25μ	Value of χ^2 is significant but the high value is due to an excess and deficit between 6 25μ and 6 75μ	Probability of χ^2 exceeding 22 435 is 0 01	Probability of χ^2 exceeding 12 851 is greater than 0 1	Probability of χ^2 exceeding 16 477 is greater than 0 5

TABLE E—*concld*

Showing the frequency distributions and the goodness of fit of curves worked out to represent the distribution of RBC diameters on various days for monkey 7

	(f)	(g)	(h)	(i)	(j)	(k)	(l)
Date of observation —	29–8–41 (4th day after injection)	30–8–41 (5th day after injection)	2–9–41 (8th day after injection)	4–9–41 (10th day after injection)	6–9–41 (12th day after injection)	9–9–41 (15th day after injection)	12–9–41 (18th day after injection)
Cell diameter	Frequency (major component)	Frequency (complete distribution)	Frequency (complete distribution)	Frequency (complete distribution)	Frequency (major component)	Frequency (major component)	Frequency (major component)
4 00							
4 25							
4 50	4 3	0 4	0 1				
4 75	14 0	2 1	0 3				
5 00	38 0	7 9	1 2				
5 25	71 9	22 3	3 4				
5 50	94 8	46 6	8 1	2 0			
5 75	87 3	72 3	15 4	6 2			
6 00	56 0	86 1	23 4	11 9			
6 25	25 0	75 5	28 5	17 1			5 9
6 50	9 8	50 7	27 9	20 5		8 6	10 6
6 75		29 6	25 3	22 6	5 9	18 4	22 8
7 00		18 6	17 8	21 0	13 6	32 4	41 3
7 25		15 5	14 5	21 7	26 7	49 6	62 0
7 50		15 2	15 5	26 0	42 1	67 8	78 0
7 75		14 6	26 6	36 6	55 7	81 7	82 0

8 00		13 0	27 2	51 6	65 0	65 2	72 0
8 25		10 5	34 6	61 4	60 9	59 2	52 0
8 50		7 8	40 2	66 4	60 7	48 5	32 3
8 75		5 2	42 2	56 0	49 2	34 6	16 6
9 00		3 1	40 6	38 7	34 0	20 4	7 1
9 25		1 7	35 3	21 8	19 4	8 7	3 5
9 50		1 4	28 2	10 1	8 4	1 9	
9 75			20 3	3 8	2 5		
10 00			13 4	1 6			
10 25			8 0				
10 50			4 4				
10 75			2 2				
11 00			1 0				
11 25			0 4				
11 50			0 2				
TOTAL	101 1	500 1	500 2	500 0	450 0	467 0	487 0
χ^2 value	10 222	12 34	11 52	20 705	39 017	23 805	21 944
REMARKS	Probability of χ^2 exceeding 10 222 is greater than 0 1	Probability of χ^2 exceeding 12 34 is less than 0 01 The bad fit is due to the distribution of 119 cell diameters of the minor component not being smooth	Probability of χ^2 exceeding 11 52 is high being greater than 0 7	Probability of χ^2 exceeding 20 705 is about 0 1	Probability of χ^2 exceeding 39 017 is very small, but the high value of χ^2 is almost due to fewer cells observed with diameter 7 75μ being made up by an excess of cells with diameters just greater and just below this value	Probability of χ^2 exceeding 23 805 is less than 0 01 the fit cannot be considered as bad since the main reason for the high value of χ^2 is a deficit of cell diameters with value 7 75μ and an excess with value 7 50μ	Probability of χ^2 exceeding 21 944 lies between 0 01 and 0 02 The excess and defect for values near 7 50μ cause the high value for χ^2

TABLE I-*a*.

Frequency distribution of red cell diameters

Monkey A

Cell diameter	Before injection, 19-3-42	Before injection, 19-3-42	After injection, 19-3-42	20-3-42	21-3-42	23-3-42	24-3-42	25-3-42	26-3-42	30-3-42	2-4-42	7-4-42
4 00						1						
4 25						0						
4 50					3	7	2					
4 75					8	13	3	1	2			
5 00			1	2	24	52	16	2	6			
5 25			1	9	75	73	32	6	12	2	2	
5 50			1	25	107	102	71	31	27	3	4	2
5 75	1	3	12	69	124	100	88	46	55	9	14	4
6 00	2	4	22	118	69	31	59	68	55	16	11	10
6 25	7	13	41	97	38	14	29	54	25	11	5	10
6 50	27	50	112	76	18	7	11	21	13	9	11	22
6 75	72	157	157	61	12	7	14	15	12	18	21	31
7 00	128	281	97	26	7	13	9	3	1	21	45	67
7 25	120	238	40	11	9	4	14	4	13	51	69	109
7 50	99	163	10	4	2	4	4	7	13	63	87	104
7 75	20	51	3	0	0	6	8	5	25	57	71	43
8 00	18	28	1	1	0	13	23	10	39	79	87	65
8 25	5	9	2	1	2	5	18	19	42	68	46	20
8 50	1	3			2	4	19	23	44	43	21	10
8 75						16	18	37	52	22	5	3
9 00						10	19	26	25	15	1	
9 25						1	12	31	12	7		
9 50						4	12	33	15	4		
9 75						6	7	29	10	2		
10 00						4	4	15	1			
10 25						1	4	6	0			
10 50						1	2	5	0			
10 75						1	1	0	0			
11 00							0	0	1			
11 25							1	3				
TOTAL	500	1,000	500	500	500	500	500	500	500	500	500	500

TABLE I-*b*

Frequency distribution of red cell diameters

Monkey 7

Cell diameter	Before injection 25-8-41	Before injection 25-8-41	After injection 25-8-41	After injection 25-8-41	26-8-41	27-8-41	28-8-41	29-8-41	30-8-41	2-9-41	4-9-41	6-9-41	9-9-41	12-9-41
4·00														
4 25														
4 50					1	8	2	7	1	2				
4 75					2	7	3	9	0	0				
5·00			2	3	6	34	30	44	9	2				
5·25			6	12	24	58	58	65	18	0		1		
5 50			14	35	52	69	87	92	53	6	2	0	2	2
5 75	1	1	28	70	76	97	94	88	75	17	12	1	2	1
6·00	1	5	55	111	75	89	85	73	83	25	6	3	5	6
6 25	1	6	49	109	68	54	45	25	61	23	11	8	9	8
6 50	9	37	77	157	48	27	25	31	54	32	25	12	13	9
6 75	19	66	124	242	67	28	29	21	46	22	21	26	25	18
7 00	71	146	75	139	30	13	19	9	24	23	21	17	27	43
7 25	120	258	44	73	28	10	14	15	14	18	28	33	57	86
7 50	127	227	20	30	10	3	3	6	15	14	24	48	77	80
7 75	51	97	3	11	7	0	2	2	7	17	30	23	43	57
8 00	53	93	1	4	4	0	4	3	6	31	58	81	66	75
8·25	28	44	1	2	1	3		4	13	35	74	81	57	57
8 50	7	12	1	1	1			2	3	39	61	47	58	26
8 75	3	6		1				2	9	44	53	59	27	18
9·00		2						1	4	41	34	35	19	10
9 25								1	3	27	20	13	9	4
9 50									2	30	14	11	4	
9 75										20	5	1		
10 00										13	1			
10 25										11				
10·50										6				
10 75										1				
11 00										0				
11 25										1				
TOTAL	500	1,000	500	1 000	500	500	500	500	500	500	500	500	500	500

TABLE II-*a*

Relative frequencies in the two components with their means, standard deviations and Pearsonian β_1 and β_2
Monkey A

Date	Days after injection	Component on the left					Component on the right				
		Number of cells	Mean	Standard deviation	β_1	β_2	Number of cells	Mean	Standard deviation	β_1	β_2
19-3-42	Before injection	500	7 161μ	0 3935μ	0 0102	3 5532					
19-3-42	Before injection	1,000	7 141μ	0 3892μ	0 0303	3 8026					
19-3-42	After injection	500	6 705μ	0 3956μ	0 0351	4 7750					
20-3-42	1st day	483	6 201μ	0 4242μ	0 0036	2 6978	17	7 325μ	0 3517μ		
21-3-42	2nd day	472	5 671μ	0 4133μ	0 0371	3 3203	28	7 223μ	0 5400μ		
23-3-42	4th day	406	5 509μ	0 4261μ	0 0570	3 9645	94	8 396μ	0 9713μ	0 0277	2 2886
24-3-42	5th day	320	5 738μ	0 4153μ	0 0301	3 5139	180	8 496μ	0 9214μ	0 0142	2 7510
25-3-42	6th day	249	6 024μ	0 4038μ	0 0282	3 3539	251	9 070μ	0 7336μ	0 0001	3 0930
26-3-42	7th day	208	5 894μ	0 4188μ	0 0018	3 1727	292	8 459μ	0 6276μ	0 0956	3 2631
30-3-42	11th day	44	6 040μ	0 3800μ			456	7 888μ	0 6321μ	0 0011	3 1177
2-4-42	14th day	38	5 888μ	0 3133μ			462	7 629μ	0 4945μ	0 0099	2 5940
7-4-42	19th day	15	5 900μ	0 2194μ			485	7 400μ	0 5026μ	0 0001	2 9643

TABLE II-*b*

Relative frequencies in the two components with their means, standard deviations and Pearsonian β_1 *and* β_2
Monkey 7

Date	Days after injection	Component on the left					Component on the right				
		Number of cells	Mean	Standard deviation	β_1	β_2	Number of cells	Mean	Standard deviation	β_1	β_2
25-8-41	Before injection	500	7 451μ	0 4360μ	0 0330	3 5218					
25-8-41	Before injection	1,000	7 386μ	0 4061μ	0 0289	3 4928					
25-8-41	After injection	500	6 591μ	0 5327μ	0 0588	3 1858					
25-8-41	After injection	1,000	6 553μ	0 5425μ	0 0067	3 2059					
26-8-41	1st day	482	6 188μ	0 5842μ	0 0102	2 3681	18	7 806μ	0 2708μ		
27-8-41	2nd day	464	5 755μ	0 4958μ	0 0064	2 7732	36	7 153μ	0 4009μ		
28-8-41	3rd day	424	5 704μ	0 4084μ	0 0040	2 6228	76	7 001μ	0 3661μ		
29-8-41	4th day	401	5 567μ	0 4098μ	0 0257	2 8035	99	7 015μ	0 6907μ	1 7091	3 9811
30-8-41	5th day	381	5 992μ	0 4512μ	0 0001	2 6932	119	7 588μ	0 7963μ	0 3414	2 3156
2-9-41	8th day	155	6 350μ	0 5281μ	0 3455	3 8005	345	8 765μ	0 8104μ	0 0122	2 6618
4-9-41	10th day	124	6 675μ	0 5236μ	0 0763	2 4069	376	8 413μ	0 5531μ	0 0217	2 8520
6-9-41	12th day	50	6 550μ	0 3500μ			450	8 185μ	0 6188μ	0 0074	2 5439
9-9-41	15th day	33	6 311μ	0 3590μ			467	7 894μ	0 6397μ	0 0174	2 4214
12-9-41	18th day	13	5 981μ	0 2402μ			487	7 694μ	0 5852μ	0 0385	2 7328

ON THE FRAGILITY OF ERYTHROCYTES

Part I

IN HYPOTONIC SALINE

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[Received for publication, October 29, 1942.]

An interesting point in connection with hæmolysis is the variation in the resistance of erythrocytes obtained from the different species of animals towards a particular hæmolysin. Speculations in this connection first arose with reference to the contribution by Rywosch (1907) who pointed out that the series obtained by writing down the names of common laboratory animals according to the order of resistance of their erythrocytes to hypotonic saline is just the reverse of that obtained by ranging them according to their resistance to saponin hæmolysis. This inverse relation between the order of resistance to saponin and that to hypotonic saline suggested the possibility of the existence of some factor which operates in one sense with respect to one lysin and in the reverse sense with respect to the other, and various suggestions were put forward as to the probable nature of that factor.

Port (1910) pointed out that the order of resistance to saponin for the red blood cells of different animals was the same as the order of their phosphoric acid contents. Hober and Nast (1914) and also Orahovats (1926) accepted the idea that the factor determining resistance was phosphoric acid. Yagi (1911) and also others have, on the other hand, contended that it was the cholesterol content of the cell which determined the resistance, but these various contentions do not seem to rest on a very sound footing. On an examination of the figures for phosphoric acid content of the red blood cells of different animals as given by Abderhalden (1895) and which Port had used in support of his thesis, it is apparent that the greater content of phosphoric acid was associated with lower resistance to saponin. All available information regarding the mechanism of saponin hæmolysis however, tends to show that saponin enters into combination with some constituent of the cell. If that be the case we should expect that the greater the amount of that cell component with which saponin is supposed to combine, the more resistant would be the cell towards saponin hæmolysis, as more of the saponin will be used up before hæmolysis takes place. This is contrary to observed facts. The same difficulty confronts us if we assume the cholesterol content to be the determining factor in regulating cell resistance. Again the resistance of corpuscles to hæmolysis by hypotonic saline solutions has been found to be independent of the cholesterol content of the corpuscles or plasma (Delas, 1933).

Ponder (1926) considered that it was the protein constituent of the cell wall which determined resistance. An examination of Abderhalden's table no doubt shows that higher figures for protein content are associated with greater resistance of the respective cells towards saponin hæmolysis, but it is difficult to see in what manner the combination of saponin and protein is effected and even assuming that saponin combined with the protein component of the cell membrane, it is not clear why greater protein content of the cell membrane should be associated with lower resistance to hypotonic saline. Ponder himself was not very happy about his suggestions. If there was any single factor, he thought, which determined the cell resistance with respect to saponin in one direction and to hypotonic saline in the reverse manner, this inverse relation should hold good for the erythrocytes of all animals and not for the particular animals examined by Rywosch. Ponder, Salsow and Yeager (1930) accordingly examined the resistance of the cells of a number of animals both to saponin and hypotonic saline but no clear inverse relation could be established.

Distilled H_2O, c c	2 0	1 9	1 8	1 7	1 6	1 5	1 4	1 3
1 per cent NaCl, c c		0 1	0 2	0 3	0 4	0 5	0 6	0·7
Concentration of NaCl, per cent		0 05	0 1	0 15	0 2	0 25	0 3	0 35
Name of animal				Per cent	Per cent	Per cent	Per cent	Per cent.
Guinea pig			+++++	90	95	90	80 80 80	40 50 60
Human			+++++	98 +++++	95 99	90 95	80 60	50 —
Monkey (rhesus)					+++++	98	+++++ 80	60 40
Dog					+++++ +++++	99 99	90 98 +++++	10 80 40
Rabbit							+++++ +++++ +++++ +++++	+++++ 80 98 98
Buffalo								+++++
Ox							+++++ +++++	80 60
Cat								+++++ +++++
Sheep							+++++	98
Goat								+++++

+++++ =
± =
— =

hypotonic saline

1 2	1 1	1 0	0 9	0 8	0 7	0 6	0 5	
0 8	0 9	1 0	1 1	1 2	1 3	1 4	1 5	
0 4	0 45	0 5	0 55	0 6	0 65	0 7	0 75	
Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Per cent	Average diameter, μ
5	–	–	–	–	–	–	–	7 88
–	–	–	–	–	–	–	–	
5	–	–	–	–	–	–	–	
–	–	–	–	–	–	–	–	7 82
–	–	–	–	–	–	–	–	
5	–	–	–	–	–	–	–	7 51
±	–	–	–	–	–	–	–	
–	–	–	–	–	–	–	–	7 13
20	–	–	–	–	–	–	–	
±	–	–	–	–	–	–	–	
40	±	–	–	–	–	–	–	6 96
60	10	±	–	–	–	–	–	
80	10	–	–	–	–	–	–	
60	10	–	–	–	–	–	–	
80	20	±	–	–	–	–	–	6 20
	+++++	80	40	±	–	–	–	
60	20	±	–	–	–	–	–	6 02
40	10	±	–	–	–	–	–	
98	80	5	–	–	–	–	–	5 81
+++++	80	10	–	–	–	–	–	
	+++++	60	10	±	–	–	–	
	+++++	10	±	–	–	–	–	
	+++++	80	40	10	–	–	–	
	+++++	80	10	–	–	–	–	
95	90	+++++	80	5	–	–	–	5 17
		80	60	40	5	–	–	
		+++++	60	±	–	–	–	
99	98	95	90	10	2	–	–	4 10
	+++++	80	40	20	±	–	–	
	+++++	90	40	10	±	–	–	
	+++++	98	10	5	±	–	–	
			+++++	60	40	5	–	

Complete hæmolysis
Doubtful hæmolysis
No hæmolysis

Again Ponder and MacLachlan (1927) found that the order of resistance towards one lysin was not the same as that towards another and a different resistance series occurred for each lysin examined From the above one would be constrained to assume that different lysins attack different constituents of the r b c or affect the same cell constituent in a different manner

The resistance of the erythrocytes towards cobra venom hæmolysis affords an extreme instance to the point Kyes (1910) determined the relative resistance of red blood cells of a number of animals to cobra venom hæmolysis and the series obtained by this method runs almost parallel to the series obtained by Rywosch with respect to saponin, excepting that cells of certain animals such as sheep, goat and ox which offer the greatest resistance to saponin hæmolysis are absolutely resistant to the action of cobra venom It is of interest in this connection to note that Faust believed the poisonous element of cobra venom to be a glucoside closely resembling sapotoxin The fact that certain cells are absolutely resistant towards a particular lysin does not fit in with the idea that the susceptibility of cells depends upon the presence of a particular cell component in greater or smaller amounts Such absolute resistance rather suggests the absence of a particular constituent or constituents either *in toto* or in an available form in the cells concerned Later, Ponder (1934) tried to get over this difficulty by supposing the cell components, or even the protein components alone to vary in nature from animal to animal and that the compounds formed when these react with different lysins are themselves different 'When enough of the compound is formed semi-permeability may be lost, but the quantity which requires to be formed (and which measures the resistance of the cell) will clearly depend on the contribution which the compound makes to the semi-permeability as a whole'

It is of course not very difficult to attribute the variation in the susceptibility of the red blood cells of different animals towards a particular hæmolysin (other than hypotonic saline) as being due to the combination of that lysin with a particular component of the cell and which varies in quality, or quantity, or in both, with the nature of the cell, but it is only when any relation is sought to be established between such resistant series and that obtained in hypotonic salt solution, that real difficulty is experienced because the mechanism of hæmolysis in hypotonic saline is of an entirely different nature, as in this case, we are not dealing with a process in which lysin is used up Judging from the light of evidence available, this inverse relation in the Rywosch series appears to be purely accidental This, however, leaves the question of the difference in fragility of red blood cells from different animals in hypotonic saline quite unsettled

Hæmolysis in hypotonic saline is commonly believed to be an osmotic phenomenon and as such, variations in the fragility of corpuscles in this medium is to be attributed either to a difference in the osmotic pressure inside the cell or to a difference in the fragility of the limiting membrane

While comparing the relative fragility of the erythrocytes belonging to different species of animals in hypotonic saline, it appeared to us that some erythrocytes known to be of very small size, such as those of sheep and goat, were very fragile, while others having bigger sizes, such as those of man, dog, etc, were far more resistant In the Table, the relative fragility of erythrocytes of some common animals and their respective average diameter as given in Hawk and Bergeim's 'Practical physiological chemistry', 10th ed, 1931, p 377, are compared

Technique for determining fragility

Different amounts of distilled water and an accurate 1 per cent solution of NaCl are put in a series of test-tubes as detailed in the Table and 0 1 c c of the blood is added to each of the tubes The contents of the test-tubes are well mixed and these are then put in an incubator at temperature of 37°C for half an hour when readings are taken Readings are in many cases checked by comparison with a colorimeter fitted with micro-cups and plungers

From the Table it would appear that the fragility of the erythrocytes of different species of animals gradually increases as there is a diminution in the value of their mean diameter This relation is not strictly proportional and in certain cases, as for instance, with respect to the corpuscles of buffalo and ox greater fragility is found sometimes to be associated with greater size of the cells and vice versa The agreement however is as close as can possibly be expected, in view of the fact that sometimes there is an appreciable divergence in the fragility of corpuscles of individual animals belonging to the same species and also that the fragility test and the measurement of the corpuscular diameter were not done simultaneously with respect to the same animal but that the fragility tests carried out with respect to the erythrocytes of certain animals in India were compared against the corpuscular diameter of animals of different breed and living in a different part of the world The Table also shows very clearly the marked irregularity in the fragility of individual erythrocytes in a given specimen of blood, some being more fragile than the others Taking our observed relation regarding the fragility and the size of the corpuscles to be correct this irregularity in fragility is an index of the irregularity in the size of erythrocytes composing the blood and the proportion of cells of different sizes may roughly be ascertained from the fragility figures

SUMMARY AND CONCLUSIONS

1 A preliminary investigation with an attempt to correlate the fragility and average diameter of the erythrocytes of several species of animals was carried out

2 The fragility of erythrocytes belonging to certain species of animals was found to vary inversely as their average diameter

Note—Circumstances have prevented the continuance of this work and this preliminary observation only is reported

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SURFACE TENSION AND HÆMOLYSIS.

BY

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[Received for publication, February 17, 1943]

HÆMOLYTIC substances are of widely different nature but some of the most active amongst them have one property in common, viz they cause an appreciable lowering of the surface tension of solutions Though the lowering of surface tension is regarded as one of the most effective methods of breaking the stroma-hæmoglobin union, this property alone is not sufficient to explain all the observed facts and the chemical nature of the substance also appears to be an important factor in this respect Lumière and Retif (1928) report that the most active hæmolysins, such as saponin and hexyl resorcinol in 1 in 20,000 dilutions, when mixed with guinea pig's blood (dilution 1 in 50 in physiological saline), gave solutions with a surface tension of 65 and 59 respectively while two commercial products 'Nekal A E M' and 'Alborit' in minimum hæmolytic concentrations of 1 in 600 and 1 in 250 respectively caused the surface tension to drop to 10 and 19 respectively, much greater hæmolytic activity being associated with higher surface tension They conclude that there is no relation between the lowering of surface tension and hæmolytic power Ponder (1924) also finds that the surface tension of saponin solutions which cause appreciable hæmolysis do not differ materially from that of water [σ in dynes for water = 65 6 and that for saponin solution (1 in 50,000) = 63 4] It is often suggested that the hæmolytic action of the soaps is due to the lowering of surface tension caused by them Ponder (1924*a*) however, could not find any definite relation between

TABLE I

Saponin (Merck's pure)—sheep's r b c 5 per cent

Serial number	Concentration of saponin in thousands	Time taken for complete hæmolysis		Surface tension
1	1/1	0′ — 14″		112
2	1/2	0′ — 17″		114
3	1/4	1′ — 15″		115
4	1/5	2′ — 8″		115
5	1/6	4′ — 17″		115
6	1/8	15 — 0		115
7	1/10	24 — 0		117
		Hæmolysin		
		2 hrs	24 hrs	
8	1/12	95 per cent	+++++	118
9	1/14	90 „ „	+++++	123
10	1/16	15 „ „	+++++	121
11	1/18	10 „ „	+++++	121
12	1/20	5 „ „	80 per cent	122
13	1/22	—	10 „ „	123
14	1/24	—	5 „ „	125 5
15	1/30	—	5 „ „	126 0

N B—Surface tension of distilled water under conditions in which these experiments were carried out varied from 158 to 161 (direct reading on the dial)

+++++ Complete hæmolysis

— No hæmolysis

the concentration of soap solutions and the surface tension The surface tension was found almost the same for all concentrations above about 1 in 30,000 and thereafter becomes greater reaching that of water at a concentration of about 1 in 100,000 In spite of such isolated findings, there is still a strong general belief that surface tension is responsible for bringing about hæmolysis in a large number of instances In view of the rather unsatisfactory state of our knowledge regarding this point, this investigation was started for the purpose of ascertaining how far lowering of surface tension is responsible for the action of some of the more well known hæmolytic agents The hæmolysis was determined according to the technique described earlier (Roy *et al*, 1940) Different concentrations of the lysin were allowed to act on 0 3 c c of washed 5 per cent sheep's r b c the total volume of 1 c c being made up with sterile normal saline The surface tension was determined by means of du Nouy's apparatus, direct readings from the dial being taken Surface tension and hæmolysis were determined simultaneously with respect to the same concentrations of the lysin and as far as possible at the same temperature

Table I shows that though the time taken for complete hæmolysis on the whole increases with the rise of surface tension of solutions, there is no proportionality between the two In concentrations of from 1 in 4,000 to 1 in 8,000, there is a rise in time of complete hæmolysis from 1'-15" to 15'-0" but at all those concentrations the surface tension remains almost stationary Moreover, saponin solutions having concentrations of 1 in 1,000 to 1 in 2,000 which cause almost immediate hæmolysis have surface tensions of 112 and 114 respec tively at which no hæmolysis takes place in the case of other hæmolysins This shows more or less conclusively that with respect to saponin at least surface tension is not the proximate cause for bringing about hæmolysis

TABLE II.

Sodium glycocholate (Merck's)—sheep's r b c 5 per cent

Serial number	Final concentration of glycocholate	Time for complete hæmolysis	Surface tension
1	1/50	12′ — 40″	95 5
2	1/100	30′ — 8″	96 0
3	1/150	43′ — 18″	96 0
4	1/200	34′ — 5″	96 5
5	1/300	37′ — 48″	94 5
6	1/400	26′ — 0″	96 0
7	1/500	20′ — 45″	91 0
8	1/800	4′ — 35″	89 0
9	1/1,000	2′ — 57″	87 5
10	1/1,200	3′ — 15″	87 0
11	1/1,600	5′ — 35″	85 0
12	1/1,800	9′ — 40″	85 0
13	1/2,000	12′ — 0″	87 0
14	1/2,500	37′ — 0″	92 5
15	1/3,000	81′ — 0″	94 0
16	1/5,000	— (21 hours)	105 0

Sodium glycocholate is one of those substances which have abnormal time-dilution curves for hæmolysis From Table II it appears that the time taken for complete hæmolysis at different dilutions gradually increases (with occasional irregularities) from 1/50 dilution to about 1/300, then it gradually decreases again to a minimum at about 1/1,000 and then increases again with higher dilutions of the lysin This maximum hæmolytic action of the substance at about 1/1,000 dilution we have repeatedly observed and therefore the observations of Ponder (1922) to that effect are corroborated In contrast to this, the surface tension values do not show any wide variations Through the whole range of dilutions from 1/50 to 1/3,000 where

the time for complete hæmolysis varies from about 3 minutes to 81 minutes the surface tension values fluctuate by only eleven units So that with this salt also surface tension does not seem to play any significant part in bringing about hæmolysis, though its low values might be a contributory factor in precipitating hæmolysis brought about by some other mechanism (to be discussed elsewhere)

TABLE III

Sodium taurocholate (Difco)—sheep's r b c 5 per cent

Serial number	Final concentration of Na taurocholate	Time for complete hæmolysis	Surface tension
1	1/100	Immediate	91 8
2	1/150	Immediate	90 5
3	1/200	0 — 5″	85 5
4	1/300	0′ — 13″	88 0
5	1/500	1 — 38″	89 0
6	1/700	7′ — 38″	87 0
7	1/900	10 — 0″	86 5
8	1/1 100	11 — 0″	84 0
9	1/1 300	26 — 0″	84 5
10	1/1 500	35 — 0″ (95 per cent) 100 — 0″ (complete)	86 0
11	1/2,000	— (3 hours)	89 0

While this salt has a typical time-dilution curve for hæmolysis, the variation in surface tension is very irregular and there is no relation whatsoever between the time for complete hæmolysis and surface tension At a dilution of 1/100 when there is almost immediate hæmolysis the surface tension is 91 8, while at 1/2,000 where there is no hæmolysis within 3 hours the surface tension is 89 0

TABLE IV

Na-oleate (Merck's)—sheep's r b c 5 per cent

Serial number	Final concentration of Na-oleate	Time for complete hæmolysis	Surface tension
1	1/200	Immediate	65 0
2	1/500	0 — 17″	64 8
3	1/1 000	1′ — 28″	64 5
4	1/2 000	5′ — 0″	64 8
5	1/4,000	5 — 30″	64 5
6	1/6,000	6′ — 0″	65 8
7	1/8,000	6′ — 30″	69 5
8	1/10 000	7′ — 0″	75 5
9	1/15,000	18′ — 0″	80 5
10	1/20 000	27 — 0″	86 5
11	1/25 000	50 — 0″	87 0
12	1/30,000	80 per cent in 2 hours	96 5

The surface tension figures in this case are undoubtedly very low but there is no proportionality between the variations in the time of complete hæmolysis and the corresponding variations in surface tension The hæmolytic behaviour of solutions of sodium oleate in relation

to their surface tension also shows that the hæmolysis is brought about by some mechanism other than the surface tension which plays only a secondary part in the process

TABLE V

Cyclamin (Merck's)—sheep's r b c 5 per cent

Serial number	Final concentration of cyclamin in thousands	Time for complete hæmolysis	Surface tension
1	1/2	Immediate	112 5
2	1/20	0′ — 5″	130 0
3	1/40	0′ — 18″	137 0
4	1/60	0′ — 35″	141 8
5	1/80	1′ — 12″	137 0
6	1/100	1′ — 20″	140 0
7	1/120	1′ — 50″	142 5
8	1/140	5′ — 8″	146 0
9	1/160	6′ — 0″	147 0
10	1/180	8′ — 40″	148 0
11	1/200	14′ — 20″	150 0
12	1/250	53′ — 0″	150 0
13	1/300	110′ — 0″	144 0
14	1/350	80 per cent in 3 hours	144 5
15	1/400	50 per cent in 3 hours	155 0
16	1/500	— (3 hours)	152 0

Said to be the most active of all known hæmolytic agents, cyclamin yields a typical time-dilution curve The relatively high surface tension values however, even for those concentrations which produce almost immediate hæmolysis and hæmolysis at great dilutions where the surface tensions approach that of distilled water, show conclusively that surface tension does not play any significant part in the mechanism of hæmolysis The surface tension curve is somewhat regular up to a dilution of about 1 in 60,000 when the curve assumes an exceedingly irregular form

COBRA VENOM

That the hæmolysis brought about by cobra venom has no relation whatsoever to the surface tension of the solutions is evident from the relatively high surface tension of a 1 in 2,000 solution of cobra venom (130, du Nouy) which causes complete hæmolysis of 3 per cent human r b c in about ½ hour Though there is a significant drop in the surface tension as a result of the lysis of the susceptible corpuscles by cobra venom or when hæmolysis takes place in the presence of lecithin, it does not appear to play any active part in the initiation of the process of hæmolysis Moreover, with Russell's viper venom which is almost non-hæmolytic, a 1 in 2,000 solution has about the same surface tension as that of cobra venom of the same strength

DISCUSSION.

Though the lowering of surface tension is considered one of the most effective means of breaking the stroma-hæmoglobin union, with none of the more well-known hæmolytic agents described above does surface tension appear to play any significant part in determining its hæmolytic behaviour It is indeed doubtful whether surface tension ever takes any part in the initiation of the process of hæmolysis, though it has a great modifying influence on the hæmolysis primarily brought about by some other mechanism For instance, when hæmolysis is brought about by a solution of one or more of the stroma components by the lysin, as in the case of cobra venom, bile salts, soaps (to be discussed elsewhere) hæmolysis is much quickened if the surface tension of the lysin is low as well Again, a rapid alteration

in surface tension of a suspension of r b c (either increase or decrease) when the corpuscles are already damaged through the solvent action of the lysin on the cell constituents may result in acceleration of hæmolysis

Summary and conclusions

1 Several well-known hæmolytic agents, such as saponin, sodium glycocholate, sodium taurocholate sodium oleate cyclamin and cobra venom, were studied with a view to find out if there were any correlation between the hæmolytic activity and the surface tension of the respective solutions

2 With none of the hæmolysins examined was surface tension found to play any effective part in the initiation of the process of hæmolysis

3 While, broadly speaking, the lowering of surface tension facilitates and its increase tends to retard hæmolysis the part played by surface tension with respect to hæmolysis is only a secondary one and sometimes an increased surface tension may be attended with more rapid hæmolysis

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VARIABILITY IN RATES OF POPULATION CHANGE, WITH REFERENCE TO INDIA, 1881 TO 1931, AND 1941 SOME STATISTICAL CONSIDERATIONS

BY

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[Received for publication, May 14, 1942]

INTRODUCTION

In considering the geographical basis of population changes in different regions of India and the manner in which change has taken place over a fairly prolonged period, particularly the half century from 1881 to 1931, certain statistical problems have presented themselves And, with a new census in India and in other lands, about to be published, the time seemed appropriate to present these notes for discussion Following an outline of the net changes found district by district in India from 1881 to 1931, the manner in which changes had occurred between these two dates, as checked by the four intervening censuses, have been examined (Geddes, 1941)

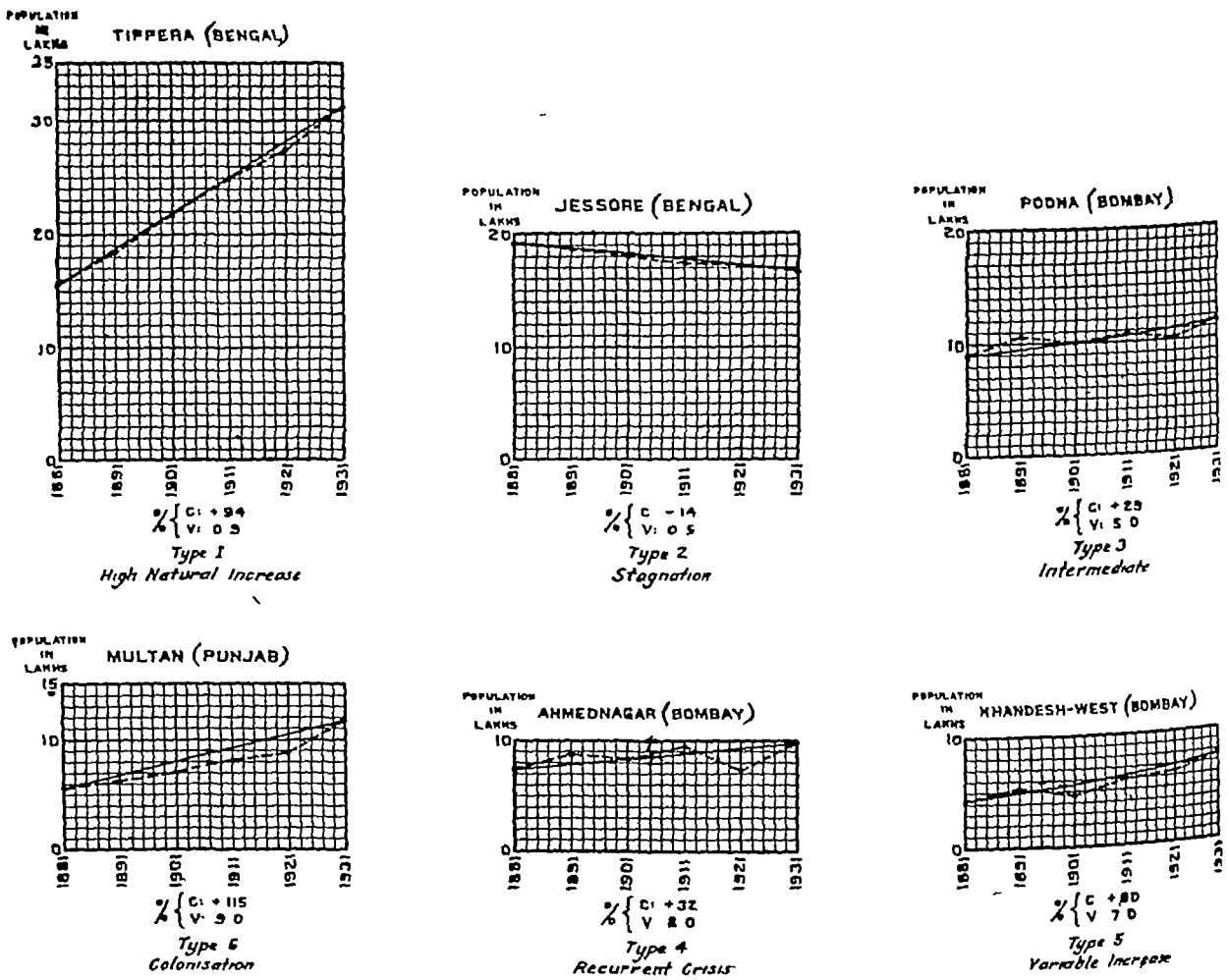
In India, striking contrasts are to be seen on the one hand between all those regions, where fair standard of subsistence and health led to relative well-being of their population and, in consequence, to a high rate of increase for half a century and, on the other hand, all regions where misery is manifest and where the net rate of increase has been low But further contrasts, clearly, are no less important In the latter class (of low net increase) there is also a striking and significant difference between certain regions to which famine comes suddenly, interrupting a period of health, well-being and increase, and others of different type which appear to escape crises but whose people suffer from chronic ill-health and a continuously low rate of survival Some criterion or index seems required to supplement the mere *net* rate of change between two census dates, by showing the character and extent of intervening changes, crises and vicissitudes This index has been described here as the *variability*

In the paper cited, it was pointed out that where a really high increase has occurred, such that at the end of fifty years from 1881 the population has doubled, it is evident that this increase, if 'natural', i e if little affected by migration, can only have been reached by means of a high increment throughout every decade Such a high, 'natural' increase, measured say at each decade, would be expressed as a smooth, rising curve An extreme increase of well over 100 to 200 or 300 per cent, on the other hand, must have been reached by help of immigration, which tends to start with a rush, giving a sharp upward bend at one or more points in the plotted curve But where, in yet another region, the population figure at the end of the period is much the same as that of fifty years before this 'net arrest' gives us no clue to what has really occurred during the half century

'In such a case two alternatives present themselves alternatives of types of which the extremes are in sharp contrast although between the two lie all manner of intermediate grades On the one hand, there may have been more or less prolonged periods when numbers rose rapidly and steadily, alternating with short periods of disaster when by drought and famine or by epidemics, the population were decimated or worse Disaster may also lead to immediate emigration, temporary or enduring, sharply diminishing the population On the other hand, a net arrest of increase may in fact be due to real stagnation From year to year, and decade to decade, the birth rate may have been equalled by a high death rate In a region of chronic misery such as this, while the people have not all simultaneously met with extreme disaster, yet they have never known health and well-being Hence, in a region in which the close of a period marked a low increase or a decline, it is imperative to distinguish whether conditions

have been much the same throughout a lengthy period or whether on the contrary there has been fluctuation or 'variability' and to what degree Variability should prove to be a telling criterion of the manner of net change (or of net arrest) throughout a period and provide a clue to the underlying causes It is essential then first to find a simple formula to express the curve which would be given by a steady rate of change from the figure of total population at the first available date (1881) to that numbered at the last (1931) We can then calculate as a fraction and percentage whatever deviation appears from the ideal curve when the actual figures are taken for the intervening censuses (1891, 1901, 1911 and 1921)'

GRAPH.



The required formula was very kindly devised by Dr A C Aitken, F R S, to whom the author wishes to record his indebtedness, adding that these notes also owe much to his commentary By Dr Aitken's method a smoothed curve was calculated without the aid of logarithms by correcting a straight line interpolation It was assumed that the smooth curve was exponential, passing through the first and last given points (i e the populations at 1881 and at 1931), and an average deviation of the given values from this curve was constructed, being expressed as a mean of the first and last values In evaluating the mean deviation it was decided to divide by 5, for it seemed inappropriate to divide either by 6, since of the six deviations the first and last are zero, or by 4 because although we force the terminal deviations to be zero we probably do this at the expense of increasing the neighbouring deviations

TABLE I

Statement showing the variation in population in certain districts in India during the period 1881 to 1931

Serial number	Name of district	Name of province	Population at the census of —					
			1931	1921	1911	1901	1891	1881
1	Tippera	Bengal	3 109 735	2 741 860	2 502 577	2,182,710	1 841,387	1,565,393
2	Jessore	Bengal	1 671 164	1 722 219	1 743 371	1 797 794	1,872,803	1,922,916
3	Poona	Bombay	1 169 798	1 009 033	1 071 512	995,330	1 007,800	901,828
4	Ahmednagar	Bombay	988 206	731 552	915 305	847,695	888,755	750 021
5	Khandesh West	Bombay	771 704	641 847	604 347	484 382	540,483	427,612
6	Lyallpur	Punjab	1 151 351	937 881	824 470	576,930	46 926	53,832
7	Multan	Punjab	1 174 000	889 328	813 357	709,297	634,538	555,516

THE IDEAL CURVE TAKEN TO BE EXPONENTIAL

It will be seen that the initial assumption was simple namely, that we can suppose that the ideal normal curve of change will be of simple exponential character (comparable to compound interest accruing momently) When it was first submitted to Dr Aitken, he was inclined to think that the idea might be taken further (Aitken 1933), but he concluded that 'least squares' would not do, for that assumes that the deviations of actual from ideal at the given points are independent of each other This would be false in practice for the deviations, as will be exemplified later, must be regarded as having some correlation In all cases of increase, forming the great majority, the ideal curve was taken to be a rising curve, while in one or two cases with no appreciable net change the 'curve' was simply a straight line Let us consider the application of this choice to India —

Given the facts, there is much to be said for this assumption for the country and the period, particularly as the figures for 1931 closed with an increase of nearly 11 per cent for India as a whole The increase for the decade to 1941, given as an increase of 16 per cent, would justify the continuation of some such assumption for the present Reasons for this condition are not far to seek Internal peace prevailed throughout India and had prevailed for long enough to permit of considerable recovery from preceding wars By 1881 the railway age had well begun in India, opening new markets for its agricultural supplies, its wheat and oil seeds, jute and cotton Famines were increasingly met by relief, sufficient, at least since the last 'great famine' of 1899-1900, to minimize death from actual starvation The standard of well-being has risen for the mass of the people, albeit slowly Measures for public health, though only a fraction of what could be desired, have increasing effect And while the death rate has fallen, for all these reasons, there seems little cause to believe that the birth rate has fallen too— whatever present tendencies may portend for the future

Not every district's population has risen however For districts and small states with a marked fall the choice lay between a curve of a quickening or a slowing rate of change The latter was chosen as corresponding most frequently to statistics and in general to the tendencies towards stabilization of hitherto falling populations

Admitting, however, that for most regions and districts of India having a rising population, an exponential curve will best form a basis or provisional 'norm' for eliciting deviations which might be described as 'abnormal', it is still worth asking whether the method is applicable to areas where the rate of change may have entered upon a new phase at some point between the first census and the last It would also be worth considering the application of the method to other countries and other cases

The logistic curve

A notable contribution to the understanding of the phenomena of change has been the concept of the logistic curve (Pearl, 1926) This is a curve like that of a geometric series or exponential function, but with a damping effect that ultimately inflicts its initial upward concavity into a downward one, so that the final value is always less than some fixed upper limit which it approaches asymptomatically

Before going further, it should be noted that the reality is almost bound to differ from any close approach to a logistic curve The fitting of a logistic curve is hardly practicable for several reasons In the first place, the statistics being limited to the comparatively short period of fifty years, the fitted curve would not be the whole curve, but only an arc , and the question of deciding in which part of the curve that arc is located, whether in the part with increasing gradient, or the part with decreasing gradient, or a part containing both kinds of gradient and the point of inflexion would introduce elements of arbitrariness Further, the deviations of population from an ideal single curve are not independent at different dates, an increase upon what has been expected, or a decrease from it, has an effect which persists, and so the deviations at consecutive census years are bound to be correlated to some extent As remarked, this prevents the ordinary application of least squares, for that is based on the assumption that deviations are independent

The above applies of course to a smooth logistic curve The changes of a population affected by crises such as famine or influenza would, however, not follow a logistic curve but be a sequence of arcs of different curves, a sequence which, as Pearl himself pointed out, may well be expected at shorter or longer intervals

Fluctuations and their lag

Differences will of course be particularly marked in tracts of scarcity or famine, where famine, economic crisis or epidemic not only lower the survival rate immediately but bring effects felt long afterwards As Mr A M Macmillan, C I E , has expressed it, in corresponding with the present writer on this subject and bearing in mind a lifetime's work in the famine zones of Western India ' In a region of fluctuating productivity the effects of say a good cycle in increasing population lag behind the causes There is, I think, an interesting relation and parallel here between the fluctuations (1) of produce, both yearly and in cycles of varying periods, (2) of cattle population and (3) of human population A fourth might be added too, in another sense, that of the trade cycle, whether as a phenomenon in itself or as a factor to be related to (1) and (2) and as affecting (3) changes of population During a period of high productivity, population tends to increase above the level which the fluctuating productivity, averaged out over cycles of years, is sufficient to maintain Then (as a result perhaps of failure of seasonable rains) seasonable current produce falls back, not merely to the average level, but to a trough lying as much below the average as the good period or peak stood above it The result is then a cattle famine, a human scarcity, or human famine In such a period of scarcity or famine there is a high death rate, postponement of marriages, and a lowering of numbers or, at least, a slowing down of increase of population In the next favourable period there is a smaller population, able to enjoy a more than average total produce The lag of such effects behind their first cause emphasizes the initial ups and downs This is very marked and is readily measurable in the case of cattle, which mature in two years instead of, as in the case of humans, in say eighteen years ' So, though not readily to be measured, the lag of effects is real in the human population and must be remembered We should note the relation of these facts and tendencies to interference with any ideal curve, or (in other words) to the maintenance of a high variability in ' famine tracts '

Interpretation of positive or negative sign

A further point in the interpretation of one or more dates of greatest deviation and of the accompanying sign (or signs), plus or minus, occurs in relation to the condition of the population at either census date If the variability of a district liable to crises is measured between two

dates both of lowered population i e just after famines or epidemics the intervening period of recovery will cause a marked upward trend in the are giving a decided deviation which will be of positive sign If, however the same population is measured between two dates both of which follow periods of recovery or accrual, an intervening crisis or crises, the deviation will bear a negative sign In either case however, the net rate of change will be low, and its accompaniment by a high percentage variability will at once indicate an unstable condition in the region, irrespective of the mathematical sign of the deviation

Thus in the Southern Deccan of India for example in districts of central and eastern Mysore the census of 1881 recorded a severe loss of population since 1871 following the terrible famine of 1877-78 In Chitaldroog district for instance the loss was nearly 30 per cent No severe crisis ensued until nearly 1921, when the pandemic of influenza just before had left its mark in a decline of population since 1911 The intervening recovery (with a 25 per cent increase from 1881 to 1921 in the district named) would give an extremely high variability, with a maximum positive deviation in 1891 or 1901 That calculated was from 1881 to 1931, by which time recovery after 1921 had had effect and the maximum deviations were still extremely positive in sign and dated 1901 and 1891 On the other hand, for a neighbouring district showing the same trends (Bellary in the Madras Deccan) I smoothed the rates of change from 1871 to 1881 (decrease) and 1881 to 1891 (increase) This not only reduced the apparent increase from 1881 but modified the deviation by its date and sign the loss recorded in 1921 giving a marked negative deviation

Methods of closer analysis

As already pointed out time only allowed to make a general or qualitative, not a quantitative, distinction between 'natural' change and change due to migration (Geddes, 1941) It was shown that in the Punjab canal colonies and in parts of Assam, immigration dominated but that from some tracts of very high net increase there was emigration while other tracts, of decline, showed little, if any, net emigration If the method here discussed is found useful it is hoped that Indian statisticians will analyse the question further

For emigration normally springs from poverty or the lure of relative affluence elsewhere, and in famines, even admitting that there is always a more or less known goal where they might find bread, the emigrants may be said to have 'fled for their lives' Immigration on the other hand comes where conditions offer the means for natural increase At the same time it must be remembered that if a population is physically and morally depressed beyond a certain point, there may be little emigration Depression has taken the place of 'pressure of population', as would seem to be the case in parts of the western delta of Bengal, with their low variability and stagnation Thus, in India migration tends to emphasize rather than distort change, while great and, still more, extreme variability makes it almost certain that there has been influx or perhaps, with decrease, an exodus

This brings us to the need of making up for the inadequacy of the net change taken between two widely spaced census dates Because of the random element involved, net change between two dates is a haphazard index and is still more inadequate as an indication of earlier conditions or as a means of forecast The variability goes far to correct the false impressions which might be derived from net change alone, being derived not from two but from six dates in a fifty-year period or more for a longer period or from more detailed data than are given by decennial counts But even the variability so far calculated still suffers from comparison with an ideal curve plotted between two random points, the population totals of two census dates Hence, to diminish the random element in net change between two dates, and combine this with variability, we may utilize a smoothed basis from which to estimate the variability Thus, as a basis, a truer impression of the prevailing rate of change over the last sixty-year period than is likely to be obtained from the first and last census alone could be obtained by taking the mean of the rate of change from 1881 to 1931 and of that from 1891 to 1941 Alternatively the means of the population enumerated between 1881 and 1891, and between 1931 and 1941 could be

taken, and the deviations estimated at census dates between these means This method should apply especially to districts with considerable variability, its use is relatively immaterial where change proceeded at a steady rate, i e where the variability is minimal, whether increase be high or low Still better would be the use of annual vital statistics after their errors, so great in most provinces of India, have been corrected to a point of fair reliability at each decennial census Given a mean for a few years at each end of the period at which to begin and end the curve, we should note a fixed number of the actual years of greatest deviation or variability At the same time the date and sign (plus or minus) of, say, the two greatest should be noted and expressed as a fraction or percentage

It will be noted that by Dr Aitken's method the deviations were averaged from the actual numbers or counts of population A drawback would seem to be that since the total population differs at each census, the deviations given, being simply numerical, did not truly express the relationship of the deviations either to one another or to the changes in population As Dr Aitken put it, the totals at successive counts are correlated, for a famine or epidemic tends not only to lower the population at the next census but to lower the succeeding totals To Dr W O Kermack, F R S E, the author owes the suggestion that this difficulty can be overcome by utilizing the rates of increase of intercensal periods These are not correlated in the same way as the absolute figures or totals Further in a smoothly changing population the decennial rates must all be equal, whereas each decennial increase would be greater, owing to the accrued total from which every increase begins Again in the variably changing populations found, deviations which are proportionately the same, would be equal, while the actual differences which occur would be strictly comparable Thus, estimates of variability could best be derived from deviations from the mean rate of change, utilizing the root mean square of the deviation

A convenient method would be to add the squares of the deviation from the mean decennial change in totals, divide the sum by one less than the number of intervals, and take the square root of the quotient as a measure of variability The coefficient of variability would be this square root divided by the mean total population, and conveniently multiplied by 100 In practice the differences of the logs of successive populations may be taken as a measure of the rate of increase (by which of course is meant the rate in terms of the actual population, the logarithmic rate) The calculations required are thus very simple ones

To test Dr Kermack's method examples were computed and compared with the previous results Most of the percentages reached were considerably higher, being generally more than double those reached previously Dr Aitken has expressed appreciation of the method and results He remarks that one need not find the higher results surprising A population figure is like a distance reached at a given time, a rate of change is like a velocity, and a coefficient based on deviations of data from a curve showing distances expected would be unlikely to be the same as a coefficient based on deviations of velocities from velocities expected

While the 'net reproduction rate' of Kuczynski has revolutionized the analysis of population trends in the Occident, where vital statistics are reliable and where vital customs fluctuate and are in course of radical change, the application of Kuczynski's method may be both more doubtful and less necessary in India, where vital statistics are so unreliable and where social customs have shown so little change up to the present This method has, however, been discussed by Lad in Mukerjee's 'Problems of modern India' (Mukerjee, 1939) Lad believes that it confirms the expectation of a generally slow increase in India on the basis of the long-term figures of change and would correct inferences derived solely from the census of 1931, with the 11 per cent increase of the previous decade, though that of 1941, with its record of 16 per cent increase, is still higher Lad points out that (from figures for 1901 to 1910) out of 1,000 females born, fewer than 500 reached the age of 15 and fewer than 250 the age of 45 (compared to nearly 800 and nearly 700 for England) Although few of these were unmarried, many are 'widows' who do not participate in parenthood The nuptial fertility rate per 1,000 married women of 15 to 40 for 1933 to 1936 was

207 to 220 similar to that of England and Wales in 1920 But in Bihar and Orissa for the prosperous period 1921 to 1931 it was only 151 The infant mortality is high and only some 2 9 survive of every four children born (70 per cent) and high mortality follows in childhood In the future however the reliability of vital statistics already so much improved in the province of Madras should improve elsewhere And even if fertility rates do not greatly change for some time to come the changing totals in regions of different variability make it important not to neglect the net reproduction

While for many tracts subject to little changed geographical conditions the index for a recent fifty-year period will give a picture broadly true of earlier history, this is not the case where a revolutionary change has taken place Thus the major crisis in the last hundred years of Bengal s health and population has been the extension of malaria in the centre and west, especially from 1850 to 1871-72, there followed the unretrieved ill-health represented by the low figures both of net change and intervening variability in these tracts from 1881 to 1931 Were the previous intercensal period included from 1872 to 1881, we should find telling contrasts in districts latterly gripped by the disease Thus Jessore with its steady decline and minimal variability since 1881 had apparently increased greatly in the first short period, according to the census from roughly one and a half to two millions (1,439 000 to 1,923,000) Even if the enumeration of 1872 was very incomplete and the increase to 1881 grossly exaggerated, the figure for 1931 of 1 671 000 would yet correspond to a net increase, or at the least to no more than a small net decline Correspondingly, the crisis should be recorded by great variability—a figure derivable if one allows for a reasonable increase, of 10 per cent, from 1872 to 1881 to eliminate the exaggeration of increase mentioned Khulna in the Sunderbans on the other hand, with its very slight variability but steady increase since 1881, was but continuing earlier conditions Hooghly, with a small net increase from 1881 (of 140 000), undoubtedly suffered a decline from 1872 to 1881, recorded as 145,000, so outweighing the whole succeeding increase—a crisis which would mark the variability no less than that of Jessore Northwards, Burdwan or Birbhum and Nadia, Murshidabad or Rajshahi would show with a similarly heightened, low to moderate increase a well-marked variability This would be moderate at the least, to great or even notable As these examples show scrutiny of the earliest census and even of early estimates of population and vicissitudes would permit of further generalizations and long-term conclusions

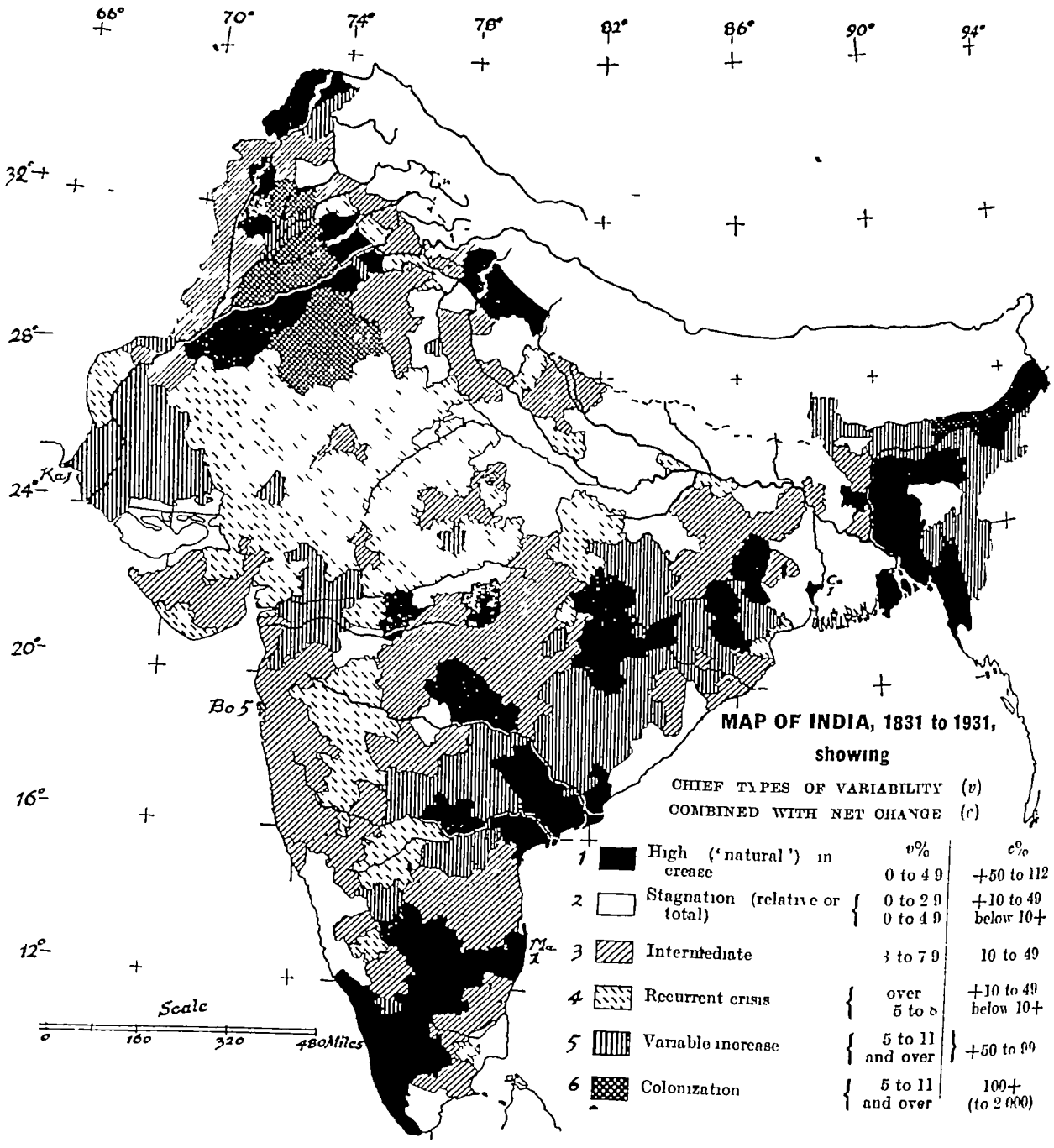
Location and geographical boundaries chief types

To geographical method particularly, the precise location of facts and factors is important Instead of merely taking certain administrative units, such as the districts of India, irrespective of the geographical and therefore probably of the demographic heterogeneity of some or many of these, those districts which partake of dual or heterogeneous characters should be subdivided into component units (in India, the subdivisions, and Talukas or Thanas) At the same time statistical caution is required in subdivisions, for the smaller the area and the population, the greater will be the random element contributing to variability and some correction is therefore required if this is to be made reliable The importance must be stressed of division along regional lines which shall distinguish tracts of forest or waste from those of cultivation and settlement, and differentiate areas liable in the past to famine from those which are famine free but liable perhaps to chronic or endemic disease For only thus can the facts be fairly ascertained and presented, and at the same time associated with the factors of their causation A paper has been completed from maps based on regional boundaries

Lastly, by classing the populations according to their combination of variability (v) with change (c), a number of types emerge When these are mapped they show well-marked correlations with the environments in which they are found The resulting map, whether a simple cartogram or a 'rational distribution map' completed by further regional analysis, permits of these types to be visualized as a single distribution, one is no

longer forced to compare two separate maps of v and c. In India these types can be classed as follows beginning with low variability (v) and high increase, as a normal condition of health and proceeding towards greater v —

MAP.



Note —While boundaries of districts and states were adhered to in this Map, as a whole, there has been some smoothing of irregularities As to values, the only serious change made has been to lower the rates of increase for forest areas of certain states on the assumption that the first census has frequently been incomplete Thus, for Bastar (19°N 81°E) the recorded increase is 169 per cent, which with 6 6, would place it in type 6 (colonization), and in fact this may be its true type Although ' increase) seems mor characteristic of the eastern forests, the author has almost no personal In Mysore state Chitaldroog (14°N 76°E) with $c + 106/v$ 6 6, should strictly borderline case of 5 and 6 due to famine of 1877 78, it has been 'smooth

CHIEF INDIAN TYPES OF VARIABILITY (v) IN CHANGE COMBINED WITH NET CHANGE (c)

A 1 v minimal to moderate, c considerable to high (e g Eastern Bengal and Travancore) or briefly *High and usually natural increase*

B 2 v minimal to slight c low to decline (e g Central and Western Bengal, Northern Bihar) or *Stagnation*

3 v slight moderate to great, but c low to moderate (e g much of western Gangetic plain of Central Provinces etc) or *Intermediate*

4 v great notable to extreme by recurrent crisis c generally low to net arrest or decline (e g 'famine tracts' of Rajputana and Deccan) *Recurrent crisis*

C 5 v great to notable (rarely extreme) c considerable to high (e g forest tracts in N-E Indian Plateau and E Ghats) *Variable increase*

6 v extreme and c (increase) extreme by revolutionary change viz by immigration (e g Punjab canal colonies and parts of Assam) *Colonization*

Details of percentages are attached to the Map Summing up it will be noticed that 1 5 and 6 show a considerable to high or extreme increase, while 1 and 2 share a low v, and 4 5 and 6 a great to extreme v

Erratum—In *Geographical Journal* p 218 para 4, last sentence should be omitted (the v for districts in Madras being calculated on the same basis as for other provinces by combining data with a smaller error only)

Were we to include earlier decades we should find a further type, where sudden disaster was followed by stagnation Although the chain of causation may be traceable to a complex of environmental factors a direct cause in India is usually an epidemic disease settling down as an endemic fever, notably malaria It may be classed D 7 v great by single crisis, c low to decline (e g Central and Western Bengal 1850 to 1931) *Epidemic-endemic*

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Ind Jour Med Res, 31, 2, October, 1943

IMMUNOLOGICAL SKIN TESTS IN LEPROSY

Part IV

THE ISOLATION OF THREE DIFFERENT PROTEIN FRACTIONS FROM *MYCOBACTERIUM LEPRÆ*

BY

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[Received for publication, April 29, 1943]

INTRODUCTION

IN Part I of this series (Dharmendra, 1942) has been reported the isolation of a protein antigen of *Mycobacterium lepræ*. It was reported that of all the fractions (protein, polysaccharide, glyceride phosphatide and wax) isolated from the bacillus, only protein is definitely antigenic.

In the same article it was stated that 'By extracting different lots of ground bacilli with weak acid, weak alkali and 80 per cent alcohol three different proteins—acid-soluble protein,

CORRIGENDUM

In paper entitled 'Surface Tension and Hæmolysis', by A. C. Roy, *Ind Jour Med Res*, Vol. **31**, No. 1, May, 1943, on page 109 Table I (centre) *read* 'Hæmolysis in' *in place of* 'Hæmolysin'.

—*Editor I. J. M. R.*

and constantly stirring while the acid is added. The nucleo-protein is allowed to settle, the supernatant is removed after centrifugalization. The precipitate is washed in distilled water, re-dissolved in weak alkali, and re-precipitated. The precipitate is finally washed in water, acetone, and ether. The washed precipitate is dried in vacuum.

Extraction with weak acid.—Ground bacilli are extracted with weak (N/20) hydrochloric acid for 10 minutes in a boiling water bath. After allowing it to cool the extract is centrifugalized. The clear supernatant extract is neutralized with N/1 NaOH and the precipitate is discarded. To the filtrate are added 3 to 4 volumes of alcohol together with some crystals of sodium acetate. The mixture is kept overnight in a refrigerator. Next morning the deposit of the acid soluble protein is separated by centrifugalization. This deposit is washed in water, acetone, and ether, and then dried in vacuum.

Extraction with alcohol.—The alcohol soluble protein was extracted by a method based on the one used by White (1932) for the isolation of such proteins from the *salmonella* bacilli. Absolute alcohol to which N/1 HCl is added at the rate of 1 c.c. of the acid to 40 c.c. of alcohol, is used for extracting the protein.

The ground bacilli are shaken with the alcohol, the mixture is warmed at 45°C. to 50°C. for half an hour, with intermittent shaking. The bacillary matter is then filtered off with the double filter paper. To the filtrate 3 volumes of ether are added, the protein is precipitated. The precipitated protein is separated by centrifugalization, washed and dried in a vacuum.

THE ANTIGENIC ACTIVITY OF THE PROTEIN FRACTIONS

All the three fractions were antigenically active, they produced early (24 to 48 hours) reaction of the 'tuberculin' type in cases of leprosy of the neural type, the nucleo-protein was most active and the alcohol-soluble protein the least.

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In the same article it was stated that 'By extracting different lots of ground bacilli with weak acid weak alkali, and 80 per cent alcohol three different proteins—acid-soluble protein, nucleo protein, and alcohol-soluble protein—have been isolated All these protein fractions produce early reactions of the "tuberculin' type in the neural cases of leprosy The methods of their isolation and the details of their antigenic activity will be considered in a future publication' This is done in the present article

THE METHODS OF EXTRACTION

Previous to extraction the bacilli were partly de-fatted and thoroughly ground The de-fattening was done by treating the bacilli with chloroform in the cold for 4 days This de fattening made the subsequent grinding easier and increased the strength of the early reaction to the bacilli, possibly by making them more friable (However, if the treatment with chloroform is prolonged beyond 4 days the strength of reaction begins to decrease) After being de-fatted the bacilli are ground in an agate mortar, or in a ball-mill, till they lose their acid fastness and bacillary form The ground bacilli are then extracted with the different solutions weak alkali, weak acid, and alcohol

Extraction with weak alkali—Ground bacilli are extracted with N/200 NaOH From the alkaline extract 'nucleo protein' is precipitated by the addition of weak (10 per cent) acetic acid using minimal amounts of acid and constantly stirring while the acid is added The nucleo protein is allowed to settle the supernatant fluid is removed after centrifugalization The precipitate is washed in distilled water re dissolved in weak alkali, and re precipitated The precipitate is finally washed in water, acetone and ether The washed precipitate is dried in vacuum

Extraction with weak acid—Ground bacilli are extracted with weak (N/20) hydrochloric acid for 10 minutes in a boiling water bath After allowing it to cool the extract is centrifugalized The clear supernatant extract is neutralized with N/1 NaOH and the precipitate is discarded To the filtrate are added 3 to 4 volumes of alcohol together with some crystals of sodium acetate The mixture is kept overnight in a refrigerator Next morning the deposit of the acid soluble protein is separated by centrifugalization This deposit is washed in water acetone, and ether, and then dried in vacuum

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THE ANTIGENIC ACTIVITY OF THE PROTEIN FRACTIONS

All the three fractions were antigenically active, they produced early (24 to 48 hours) reaction of the 'tuberculin' type in cases of leprosy of the neural type, the nucleo-protein was most active and the alcohol-soluble protein the least

The isolation of the three antigenic protein fractions from *Myco leprae* had aroused some hope that one of these fractions might be specific for *Myco lepra* This hope was strengthened by a comparison with the results obtained with the three similar fractions of the allied organism of rat leprosy *Mycobacterium leprae muris* Both the acid and the alcohol-soluble fractions of both the bacilli produced a reaction in cases of the 'neural' type and no reaction in cases of the 'lepromatous type of leprosy, these two fractions from the two bacilli do not therefore seem to differ antigenically However, the nucleo proteins from the two bacilli behaved differently the nucleo-protein of *Myco leprae* produced a reaction in only the 'neural cases, while the nucleo-protein of *Myco leprae muris* produced reactions in both the 'neural and the 'lepromatous' cases of leprosy Thus the nucleo-protein of *Myco leprae* appears to be antigenically different from the nucleo protein of *Myco leprae muris*

Testing the protein fractions for specificity

The best method for demonstrating the specificity of any of the fractions was to test persons not exposed to leprosy with these fractions If any one of the proteins were found to give negative results in a vast majority of non-contacts, a diagnostic skin-test for leprosy infection could be evolved These fractions were therefore tested in some Punjab villages where there is no leprosy and where the chances of contact of the population with cases of leprosy are very remote

The results obtained did not fulfil the hope of finding of at least one of the fractions to be specific, positive results in non-contacts were seen with all the three fractions The different protein fractions were used in a dose of 0 01 mg for the test, the incidence of positive results in non-contacts was highest (75 per cent) with the nucleo-protein, and lowest with the alcohol soluble protein (30 per cent), with the acid-soluble protein it was 60 per cent This difference in the incidence of positive results was in accordance with the observations in cases of leprosy that in this dose the nucleo-protein produced the strongest reaction and the alcohol-soluble protein the weakest

The above results in non-contacts had shown that none of the three protein fractions were specific for *Myco leprae* Nevertheless, a comparison of the results in cases of leprosy with the similar protein fractions of *Myco leprae* and *Myco leprae muris* had suggested, as reported above, that the nucleo-protein fraction of *Myco leprae* was not shared by *Myco leprae muris* and might be the specific fraction It was possible that the observed lack of its specificity was caused by some change that had taken place in it during its extraction There is some evidence that the use of alkali for extracting nucleo-protein changes the immunological properties of the nucleo-protein, the phosphate-buffer method of Heidelberger and Kindall (1931) was therefore used later to extract the protein in a more natural form The method of isolation of this fraction and the results obtained with it are stated below —

Extraction of nucleo-protein by the phosphate-buffer method —The ground bacilli are shaken thoroughly with a small quantity of acetate buffer at pH 4 0 to remove the polysaccharides The mixture is centrifugalized, the residue is separated and extracted for protein, it is ground in a mortar with M/15 phosphate-buffer at pH 6 5, and is later thoroughly shaken with the same solution The mixture is centrifugalized From the clear supernatant the protein is precipitated by glacial acetic acid, the acid is added gradually in small amounts till the resulting precipitate begins to flock The precipitate is allowed to settle, is separated after centrifugalization, and is washed and dried

Antigenic activity of the phosphate-buffer-extracted nucleo-protein

In most cases of leprosy of the neural type this fraction produced definite early (24 to 48 hours) reactions in 0 01 mg and 0 002 mg doses In non-contacts in Punjab villages the incidence of positive results with 0 01 mg was 37 per cent and with 0 002 mg 5 per cent As reported above, with alkali-extracted nucleo-protein, in 0 01 mg doses, this

percentage had been 75 per cent Thus the improvement in methods of extraction had markedly reduced the incidence of positive results in non contacts, by reducing the dose there was a further marked reduction in the incidence of positive results

Conclusions

With the improved method of extracting the antigen the percentage of positive results in non contacts has fallen markedly a specific test for leprosy infection has, however, not yet been evolved Nevertheless the work done so far gives clear indications of the existence of a specific antigen in *Myco lepræ* and encourages further attempts to isolate it more completely and in a more natural form

Summary

1 Three protein fractions have been isolated from the leprosy bacillus These fractions are the nucleo protein the acid soluble protein and the alcohol soluble protein The nucleo-protein fraction has been isolated by two different methods extraction with alkali and with a phosphate-buffer at pH 6 5

2 All the protein fractions produce allergic skin reactions of the 'tuberculin' type in cases of leprosy of the neural type

3 Comparative tests in cases of leprosy with similar fractions of the rat-leprosy bacillus indicated that while both the acid and the alcohol soluble proteins of the two organisms might be similar the nucleo proteins from them differed antigenically

4 The isolation of three different antigenically active protein fractions of *Myco lepræ* together with the fact that one of these fractions was found to be different from a similar fraction of an allied acid-fast organism, had aroused hope that this particular fraction (the nucleo protein) might be specific for *Myco lepræ*

5 The tests with the different fractions in non contacts however did not show that any of the fractions was specific This lack of specificity of the nucleo protein fraction might have been caused by some changes taking place in it during extraction This view is supported by the fact that the incidence of positive results in non contacts with phosphate buffer-extracted nucleo-protein is markedly lower than with the alkali-extracted nucleo protein

6 With improvements in the methods of isolating the nucleo-protein the incidence of positive results in non-contacts has been markedly decreased A specific antigen giving uniformly negative results in non-contacts has not however yet been isolated The work so far gives an indication of the presence of a specific antigen in *Myco lepræ* and encourages further attempts to isolate it in a more natural form

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Ind Jour Med Res, 31, 2, October, 1943

IMMUNOLOGICAL SKIN TESTS IN LEPROSY

Part V

A BACILLARY ANTIGEN STANDARDIZED BY WEIGHT

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INTRODUCTION

THE isolation of a protein antigen from *Myco leprae* (Dharmendra, 1942) and the fact that it produced results of the same significance as the Mitsuda antigen led to the suggestion (Dharmendra and Lowe 1942) that for carrying out skin tests in leprosy the isolated antigen could, with great advantage replace the ordinary *lepromin* of Mitsuda The advantages of using the isolated antigen are (1) the use of a pure antigen of a known chemical nature, which can be accurately standardized by weight, (2) the results are obtained in 24 hours instead of 3 weeks or more, and (3) undesirable late reactions ulcerations etc, not uncommonly seen with the Mitsuda reaction, are avoided

As reported in the preceding article three different proteins from *Myco leprae* have been isolated, it was hoped that one of these fractions would be specific, but so far attempts to demonstrate such specificity have failed

The lack of the specificity of any of the fractions raised the question whether the labour and special technique involved in isolating the protein fractions for performing the skin test was justifiable, and whether a standardized antigen could not be produced, that would retain most of the advantages of the isolated protein antigen, but would be simpler in preparation With this purpose, study was resumed of the whole bacilli separated from leprous nodules In cases of the neural type of leprosy, partly de-fatted bacilli were found to produce a marked early (24 to 48 hours) reaction of the 'tuberculin' type, and a slight late reaction of the nodular type The partly de-fatted bacilli were therefore considered to be very suitable for preparing an antigen standardized by weight of the bacterial powder

THE PREPARATION AND STANDARDIZATION

The bacilli are separated from the leprous material by the chloroform method described (Dharmendra, 1942) Since it was found that further treatment of these bacilli with chloroform enhanced their antigenic activity, a slight modification was introduced in the method previously described for the isolation of the bacilli

Pieces of lepromatous material, usually nodules cut from ears, are autoclaved and then ground in chloroform in a glass mortar The chloroform is pipetted off The grinding in chloroform is repeated till a smear from the remaining tissue is almost free from bacilli All the lots of chloroform used in grinding are pooled and the remaining tissue is discarded (A smear from the pooled chloroform shows bacilli in very large numbers and the absence of tissue cells or débris) The pooled chloroform extract is stored in a refrigerator for 4 days At the end of this period the chloroform is completely evaporated on a water bath, the residual substance consists of lipoids and bacilli The residue is then suspended in ether and the ethereal suspension is centrifugalized in a refrigerator The deposit consists of bacilli To remove the lipoids more completely, the bacillary deposit is again suspended in ether, the suspension is centrifugalized and the deposited bacilli are separated and dried in vacuum smears made from the dried powder show only bacilli and no tissue

Standard *lepromin* is prepared by suspending 1 mg of the dry bacterial powder in 10 c c of 0 5 per cent carbol-saline The suspension is made by putting the powder in a mortar, adding a few drops of N/10 NaOH grinding with a pestle and adding the requisite amount of carbol-saline

THE DOSE OF, AND THE REACTION TO, THIS STANDARD *lepromin*

The routine dose of the standard *lepromin* is 0 1 c c (i e 0 01 mg of the bacterial powder) injected intradermally In this dose, this preparation is capable of producing marked early reactions (24 to 28 hours) and slight but definite late reactions (2 to 3 weeks)

The early reaction is characterized by the appearance of a definite area of erythema, accompanied by definite œdema and thickening of the erythematous area The thickened erythematous area varies from 10 mm to 30 mm in diameter, occasionally more, the average being 15 mm

The late reaction is seen 2 to 3 weeks after the injection, i e slightly earlier than the reaction produced by the injection of the ordinary *lepromin* The late reaction is considerably less marked than that produced by the ordinary *lepromin* usually it consists of a small nodule from 2 mm to 4 mm in diameter, occasionally the nodule is bigger As a rule, the nodule-formation is not accompanied by ulceration, rarely, however there may be some ulceration

The standard *lepromin* was tested in 1 in 5 and 1 in 10 dilutions and was found to be still active in those dilutions, although the reactions were weaker, especially the late reactions (0 1 c c of the 1 in 10 dilution of the standard lepromin contains 0 0001 mg of the bacterial protein, the active principle It appears therefore, that the proteins of the leprosy bacillus are highly active)

THE RESULTS OBTAINED WITH THIS STANDARD *lepromin*

This standard *lepromin* has been tested both in cases of leprosy and also in healthy persons in whom chances of exposure to leprosy are very remote, with the following results —

(i) *The results in cases of leprosy* —In cases of leprosy it produces results of the same significance as those produced by the ordinary *lepromin* and by the active protein fraction isolated from the bacilli in the vast majority of the neural cases it produces marked early and slight but definite late reaction, in the vast majority of the lepromatous cases it produces no reaction early or late

(ii) *The results in non-contacts* —Tests were done in healthy persons living in an area where there is no leprosy, and in circumstances which make it highly improbable that they have ever had contact with cases of leprosy Healthy adults were tested with the routine dose (0 01 mg of the bacterial powder) and with a 1 in 5 dilution (0 002 mg of the bacterial powder) In these doses the incidence of positive results (early reactions) was 30 per cent and 3 per cent respectively

This preparation in 0 002 mg dose was tested in children, in whom an even lower incidence of positive results was to be expected, a definite positive result (early reaction) was seen in only 1 of the 170 children tested These results were very encouraging and it was considered that by regulating the dose of the antigen it might be possible to make the test (with this antigen) more 'specific' However, in some of the 'negative' cases there was a slight erythema and induration around the point of injection the reaction in these cases could not be considered positive, but it was felt that in the search for a specific test for leprosy infection, attention should be concentrated on antigens in solution which do not produce such reactions in the 'negative' cases

THE OTHER METHODS OF PREPARING STANDARDIZED *lepromin*

Till recently, no method for accurately standardizing *lepromin* was available The only precaution taken to ensure some sort of uniformity consisted in keeping a constant proportion between the weight of the lepromatous material and the saline used to suspend it Muir (1933), in addition, attempted a rough standardization by making a comparison of bacillary concentration in freshly made material and the material that had given satisfactory results, no actual bacterial count was possible

During the past 2 years, various attempts have been made at standardization of the preparations used for the *lepromin* test Apart from the preparation described in this paper the following preparations have been recommended —

(1) A fine suspension of leprosy bacilli in saline, obtained from the leprous nodules and standardized by making a bacterial count by Breed's method (Dharmendra, 1941)

(2) A solution of the protein antigen of the leprosy bacilli standardized by weight of the antigen (Dharmendra 1942)

(3) A suspension of leprosy bacilli, separated from the other tissues of the nodule and standardized by weight of the dried isolated bacilli (Fernandez, 1941)

Fernandez (*loc cit*) separates the bacterial powder from a suspension of leprous material in water, by taking advantage of the difference in densities between the lepra bacilli and the tissues He makes a watery suspension of the leprous nodules with the help of grinding Sodium chloride is added to this suspension to bring its density to 1 050, and it is then centrifugalized Most of the tissue is deposited and the majority of the bacilli remain suspended in the fluid which is pipetted off Alcohol is then added to the separated fluid to bring down its density to 0 950 The fluid is then centrifugalized and the majority of the bacilli are deposited at the bottom The bacillary deposit is dried in a vacuum and ground to a fine powder A 1 per cent suspension by weight of this powder is prepared and further dilutions of 1 in 10, 1 in 100 and 1 in 1,000 are made from this suspension Fernandez reports that this preparation produces reactions similar to those produced by *lepromin* prepared according to Muir's and Hayashi's methods

A COMPARISON OF THE VARIOUS STANDARDIZED PREPARATIONS

Including the preparation described in this article there are four preparations to be considered Three of the preparations are suspensions of leprosy bacilli and the fourth is a solution of the protein antigen isolated from these bacilli

The isolation of the protein antigen entails extra laboratory work and the use of a special technique The advantages of the use of the isolated antigen are that we use a pure and refined antigen, and that by its use late nodular reactions are altogether eliminated, since it produces an early reaction only The isolation of a specific protein fraction producing positive results in cases and contacts, and negative results in non-contacts, would have justified the extra work involved in the preparation of the protein fraction This has, however, not so far been possible It is therefore considered that for routine use, in the present state of our knowledge, it would be advantageous to have a standard antigen, retaining most of the advantages of the isolated protein but being simpler in preparation

A comparison may, therefore, be made of the suspensions made by the three different methods and the reactions produced by them The following points may be included in the comparison the ease of preparation, the yield, the purity, the accuracy of standardization, the keeping properties and the reactions produced —

(*a*) *Ease of preparation* —In a reasonably well-equipped laboratory all the three preparations can be prepared with about equal ease

(*b*) *Yield* —The grinding of leprous nodules in saline, for making a suspension, does not extract all or even most of the bacilli The extraction of the nodules with chloroform does remove most of the bacilli and the yield is therefore much greater

The yield of the bacterial powder with the Fernandez method was found to be much less than with the chloroform method From the same lot of nodular material divided into two equal portions, bacilli were isolated (*i*) by the chloroform method, and (*ii*) by the Fernandez method The yield of bacilli by the chloroform method was about three times the yield by the Fernandez method

(*c*) *Purity* —The suspension prepared from the nodules contains some fine tissue, the bacterial powder prepared by the chloroform method is practically free from tissue, the bacterial powder prepared by Fernandez method contains much less tissue than the saline suspension, but is not as pure as the chloroform-treated powder

(*d*) *Standardization* —The saline suspension can be standardized only by a bacterial count, this method has various limitations The suspensions made from the bacterial powder (obtained by either the chloroform or the Fernandez method) can be accurately standardized by weight of the bacterial powder

(*e*) *Keeping properties* —Suspensions in saline are likely to deteriorate with keeping The bacterial powders should keep much better, a fresh suspension can be made at the time of making the tests

(*f*) *Reactions produced* —All the three suspensions produce both early and late reactions in a vast majority of the cases of leprosy of the neural type, and no reaction, early or late, in a vast majority of cases of the lepromatous type The intensity of these reactions, however, varies with the different preparations

The bacillary suspension made from the leprous tissue produces well-marked early and late reactions, the suspensions made from isolated bacilli produce slightly more marked early and considerably less marked late reactions

The intensity of reaction of the suspensions prepared from isolated bacilli varies slightly with the method of isolation The suspension of the bacilli prepared by the chloroform method produces stronger reactions, particularly the early reactions This conclusion was arrived at as a result of the following experiment —

Equal weights (1 mg) of bacilli, isolated by the two different methods from two portions of the same lot of leprous nodules, were suspended in equal amounts of carbol saline 0 1 c c of the two suspensions (containing 0 01 mg of the powder) were injected into patients of the neural type of leprosy Both the preparations produced both the early and the late reactions The suspension made from the bacilli obtained by the chloroform method, however, produced stronger reactions particularly the early reactions

Thus the chloroform treated bacterial powder is, weight for weight, more potent than the powder obtained by the Fernandez method The difference in the potency of the two preparations is explainable by the fact that, weight for weight, the chloroform treated bacterial powder contains more protein antigen, since the inactive lipoids have partly been removed from it Moreover, the bacterial powder prepared by the chloroform method is more free from fine tissue than the powder prepared by the other method

Conclusion

From a discussion of the relative merits of the different standardized preparations used for carrying out the *lepromin* test, it should be clear that a suspension made from isolated bacilli has definite advantages over the one made from the leprous nodules themselves

Of the two methods described for the isolation of the bacilli, the chloroform method appears to be the method of choice

Summary

1 A method of preparing standard *lepromin* from dried and partly de-fatted leprosy bacilli is described The bacilli are obtained by extracting the nodules with chloroform, storing the chloroform extract for 4 days in a refrigerator and then evaporating it, suspending the residue in ether and centrifugalizing the ethereal suspension in a refrigerator The standardization is done by weight of the bacterial powder 1 mg of the powder being suspended in 10 c c of 0 5 per cent carbol-saline, and 0 1 c c of this suspension being used for the test

2 This preparation like the ordinary *lepromin* prepared directly from the leprous tissue, produces both early and late reactions in the cases of the neural type of leprosy and no reactions, early or late, in cases of the lepromatous type However, with this preparation the early reactions are stronger and the late reactions considerably weaker than the corresponding reactions produced by ordinary *lepromin* This is considered to be an advantage

3 The other advantages of this preparation over the ordinary *lepromin* are the use of a more refined material, more accurate standardization and the better keeping properties of the powder (the *lepromin* suspension is apt to deteriorate on keeping)

4 It is considered that this standard *lepromin* prepared from partly de-fatted bacilli retains most of the advantages of the protein antigen isolated from the bacilli, the extra labour and special technique involved in isolating the protein are eliminated

5 A comparison is made of the bacterial powder obtained by the chloroform method with the powder obtained by centrifugalizing a suspension in distilled water of leprous tissue at different densities (Fernandez method) With the chloroform method the yield of bacilli is about three times as great, and, weight for weight, the chloroform-treated bacterial powder is more potent than the one obtained by the other method

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Ind Jour Med Res, 31, 2, October, 1943

BACTERICIDAL ACTION *IN VITRO* OF SULPHANILAMIDE AND SULPHAPYRIDINE ON *MYCOBACTERIUM LEPRÆ MURIS*

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[Received for publication March 22 1943]

Introduction

The sulphanilamide group of drugs has been shown to possess a bacteriostatic or bactericidal action on a large variety of pathogenic micro organisms such as *pneumococci, streptococci meningococci, gonococci*, etc The action is not confined to the *cocci*, the drugs are known to have an action on many other groups of organisms such as the *coliform* bacilli and the organisms of the *brucella* group Some workers have found that some of the drugs possess an inhibitory action on the development of experimental tuberculosis in guinea-pigs, some other workers however have failed to demonstrate this inhibitory action

It is possible that these drugs have some bacteriostatic or bactericidal action on *Mycobacterium leprœ*, the causative organism of human leprosy If some of them do possess any such action they may have a place in the treatment of leprosy

In the absence of a method of cultivation of *Myco leprœ* and of a laboratory animal susceptible to the disease, it is difficult to study the effect of the drugs on this organism The study can, however, be made on the allied organism of rat leprosy, *Myco leprœ muris* *Myco leprœ muris* also has not been cultivated, but here we have a susceptible laboratory animal—the white rat Such a study should include (*a*) a study of the action of these drugs *in vitro* and (*b*) the action *in vivo* The results of the first part of this study are reported here Two preparations have been used in this study, sulphanilamide and sulphapyridine (M & B 693)

Preliminary experiment

Technique —A suspension of rat-leprosy bacillus was prepared from tissues of white rats suffering from experimental rat leprosy

The suspension was divided into five portions sulphapyridine (M & B 693) was added to two portions, to give a dilution of 1/1,000 in one portion and of 1/10,000 in the other Sulphanilamide was similarly added to other two portions To the remaining portion no drug was added but normal saline was added to make its volume the same as that of each of the other portions

The above five portions were divided into two parts each, thus there were two series of the five differently treated portions of the same suspension

The bactericidal or bacteriostatic action *in vitro* of sulphanilamide drugs on the various organisms has so far been demonstrated at 37°C For the purpose of the present study, there would have been a definite advantage if the drugs were found to be active at a low temperature as well as at 37°C , this would have facilitated the inclusion of untreated suspensions in the experiments as controls The bacterial suspension prepared from the tissues of rats is not absolutely free from secondary organisms , its incubation at 37°C for 48 hours is likely to make the untreated suspension unsuitable for use because of the growth of these contaminating organisms

One series of suspensions was accordingly stored in an incubator at 37°C for 48 hours The other series was stored in a cool room at about 4°C for 96 hours

After storage, 10 batches of 6 rats each were injected with the 10 portions of the suspension These rats were watched for 9 months for the development of generalized rat leprosy Whenever a rat died, a post-mortem was done, macroscopic findings recorded, and smears made from inguinal glands, liver, omentum and spleen , these smears were stained by Ziehl-Neelsen's method and examined for acid-fast bacilli The animals surviving at the end of 9 months were sacrificed and examined

Results —All the rats injected with (*a*) the untreated suspension stored at 37°C , (*b*) the suspension treated with a 1/10,000 dilution of sulphanilamide and stored at 37°C , and (*c*) suspension treated with 1/10,000 dilution of M & B 693 and stored in cold, died within 2 days of receiving the injections This was probably caused by growth of contaminating organisms in the suspension

About half the rats injected with the other portions of the suspension died within 4 months of being injected This period is usually not sufficient for the development of generalized rat leprosy The findings made in only those experimental animals which lived for 4 months or longer after the injection are therefore considered in Table I —

TABLE I

Bacterial suspension treated with	Stored at 37°C for 48 hours		Stored in the cold at 4°C for 96 hours	
	Number of rats lived for 4 months or longer	Number of rats suffered from generalized rat leprosy	Number of rats lived for 4 months or longer	Number of rats suffered from generalized rat leprosy
M & B 693 1/10,000	2	*Nil*	0	
M & B 693 1/1,000	3	*Nil*	1	1
Sulphanilamide 1/10,000	0		1	1
Sulphanilamide 1/1,000	5	*Nil*	3	3
Controls untreated suspension	0		4	4

In Table I it is to be noted that none of the rats injected with suspensions stored at 37°C suffered from rat leprosy, whereas all the rats injected with suspensions stored in cold suffered from generalized rat leprosy

Conclusions —The following tentative conclusions were drawn from the above findings —

1 M & B 693 —The drug has a bactericidal action on rat-leprosy bacillus, in 1/10,000 and 1/1,000 dilutions if allowed to act on the bacillary suspension at 37°C It has no such action in the cold

2 Sulphanilamide —The drug has a bactericidal action on rat-leprosy bacillus in a 1/1,000 dilution at 37 C , the effect of a 1/10,000 dilution could not be ascertained Like M & B 693, it has no bactericidal action in the cold

3 The untreated suspension —The untreated suspension is not suitable for injection, after storage at 37°C for 48 hours , this is due to growth of contaminating organisms The suspension remains suitable and infective after storage in the cold for 96 hours

FURTHER EXPERIMENTS

In view of the above findings it was decided, in further experiments, to use a 1/1,000 dilution of the drugs, and to let it act on the bacterial suspension at 37°C for 48 hours To exclude

the possibility of the non infectivity of the stored suspension being caused by the storage, it was desirable to include in the experiment a batch of rats injected with bacterial suspension, untreated with the drugs but treated in a way that would make possible its storage at 37°C for 48 hours without making it unsuitable for use For this purpose the suspension was treated for about half an hour with half its volume of 15 per cent sulphuric acid to give a final strength of 5 per cent of the acid, it was neutralized with 5 per cent caustic soda and then stored at 37°C for 48 hours Injections of this suspension produced generalized infection in rats

Technique —The following method was adopted for further experiments The bacterial suspension prepared from tissues of rats suffering from experimental rat leprosy was divided into three portions one portion was treated with 1/1 000 sulphapyridine, another with 1/1,000 sulphanilamide and the third with 5 per cent sulphuric acid and then neutralized All the three portions were left in the incubator at 37°C for 48 hours

After 48 hours three batches of rats were injected, one batch with each of the three different portions Each rat was given 1 c c of the suspension, 0 5 c c intraperitoneally and 0 5 c c subcutaneously into thigh

Whenever an injected animal died a post-mortem was done and the macroscopic and microscopic findings recorded Several animals lived for more than a year after being injected

Results —Sixty-four rats were injected with the bacterial suspension treated with either sulphapyridine or sulphanilamide and 20 with the acid-treated suspension to act as controls About half the rats died within 4 months of receiving the injections Since this period is not usually sufficient for the development of generalized infection, these animals are ignored, and only the findings made in the animals that lived for more than 4 months after the injection are considered in Table II —

TABLE II

The bacterial suspension treated with	Number of rats lived for 4 months or longer	Number of rats showed generalized rat leprosy	Microscopic findings
Sulphapyridine (M & B 693) 1/1,000	18	0	In 1 rat smears from inguinal gland spleen and omentum contained a few bacilli In 17 rats all smears negative for acid fast bacilli
Sulphanilamide 1/1,000	15	0	In 1 rat smears from omentum contained a few bacilli In 14 rats all smears negative for acid fast bacilli
5 per cent sulphuric acid (controls)	10	10	Smears from lymph glands, liver, spleen and omentum showed large number of acid fast bacilli in all the animals

The finding of a few organisms in 1 rat in a batch is not considered to be of significance, the presence of a few bacilli may be caused by a mere persistence of the dead organisms, even dead bacilli are known to have extraordinary powers of persistence in the tissues of living animals

The above findings indicate that sulphapyridine and sulphanilamide have a definite bactericidal action *in vitro* on the rat-leprosy bacillus The negative findings in the rats injected with the bacterial suspension treated with the drugs, could not be attributed to a mere inhibitory effect of the drugs, since many of the experimental animals lived for a much

longer period than that necessary for the development of generalized leprosy, of the 33 rats, 7 lived for 4 to 8 months, 16 for 8 to 12 months and 10 for 12 to 16 months after being injected with the drug-treated suspension

Summary and conclusions

1 A study has been made of the action *in vitro* of sulphapyridine (M & B 693) and sulphanilamide on the rat-leprosy bacillus

2 A preliminary experiment suggested that the drugs have a bactericidal action if allowed to act on the bacterial suspension at 37°C for 48 hours, but that in cold (at 4°C) they have no such action, even if allowed to act for double the time (96 hours)

3 A suspension of rat-leprosy bacillus was prepared from the tissues of white rats suffering from experimental rat leprosy This suspension was divided into three portions one portion was mixed with sulphapyridine to give a dilution of 1/1,000 of the drug, another portion with sulphanilamide in the same dilution, and the third was treated with 5 per cent sulphuric acid and then neutralized All the three portions were left in an incubator at 37°C for 48 hours Injections were then made in three batches of rats

4 The suspensions treated with the two drugs did not produce the disease in the injected animals The acid-treated suspension produced a generalized rat leprosy The non-infectivity of the drug-treated suspensions was therefore not caused by the mere storage of the suspension at 37°C for 48 hours

5 Thus, sulphapyridine and sulphanilamide, in a dilution of 1/1,000, possess a bactericidal effect *in vitro* on the bacillus of rat leprosy if allowed to act on the organism at 37°C for 48 hours Sulphapyridine was found to possess this property in a dilution of 1/10,000 also, results for a similar dilution of sulphanilamide are not available

Ind Jour Med Res **31**, 2 October 1943

STUDIES ON THE GLYCOLYTIC BREAKDOWN OF GLUCOSE IN VEAL INFUSION

BY

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[Received for publication, April 7, 1943]

GLYCOLYSIS is the term applied to the enzymic breakdown of the carbon chain of the carbohydrates in absence of oxygen All anærobic fermentation and anærobic degradation of carbohydrates in animal cells are glycolytic process and the term glycolysis is in general applied to the production of lactic acid in the animal organism

At present it cannot be stated with certainty what phases occur in the course of glycolysis as every chemical investigation of the intermediate stages of this kind is dependent on the disturbance of the co ordination of the cells, destruction of the cells and so on and processes may occur which do not take place in intact cells It can at least be taken as certain that anærobic degradation of carbohydrate in the animal cells leads to the production of sarcolactic acid and other C_3-compounds In the ærobic condition as shown by Meyerhof (1926, 1930), a reversible reaction takes place and the products of breakdown are completely or partly re-converted into glycogen This phenomenon as shown by Meyerhof later on, does not take place in *tissue extract* and it is possible to work with meat extract in presence of oxygen Cori and Cori (1936, 1937) and Embden *et al* (1933) have shown that the glycolytic process is initiated by the formation of glucose-1-phosphate from glycogen and inorganic phosphate Sullmann (1939) has shown that lactic acid formation can also take place from glucose or fructose in place of glycogen The substrate in either case should contain inorganic phosphate and an enzymic system in which adenylic acid acts as a co enzyme Another enzyme then converts this 1-phosphate to glucose-6-phosphate and then through other intermediate products to lactic acid

Lactic acid commercially available at present, is either in the form of U S P syrup which usually exhibits a low optical activity, corresponding to the excess it happens to contain, which is variable, of one or the other optical isomer or is the expensive zinc sarcolactate The method described below makes it possible to obtain quickly and easily and at low cost large amount of sarcolactic acid Since glycogen, the precursor of lactic acid in muscle, is more or less limited, attempts were made to use glucose in place of glycogen as the parent substance for the production of sarcolactic acid in meat infusion and a work in this direction was undertaken

EXPERIMENTAL

Veal was infused with two parts of water and various concentrations of glucose were added to it and the amount of glucose left in the solution and lactic acid formed was recorded pH of the substrate was adjusted to 7 0 using a solution of 4 per cent sodium bicarbonate and the temperature was maintained at 30°C Temperature higher or lower than this was found to be unsuitable No outside addition of inorganic phosphate, Mg or co-enzyme was made Infusion was always made within 2 hours after the animal was killed The results are recorded in Table I —

TABLE I

Time in hours	Glucose added in mg per 100 c c	Glucose left unconverted in mg per 100 c c	Lactic acid formed in mg per 100 c c
12	*Nil*	10 to 15	150 to 200
	250	40	200
	500	40	250
	850	50	300
	1,000	150	400
	1,500	400	400

In this and all subsequent experiments, the infusion after desired period of reaction was steamed for ½ hour and filtered through lint Glucose was estimated according to Hagedorn and Jensen (1923) and lactic acid according to Friedemann and Graeser (1933) after removing sugar by $Ca(OH)_2$-$CuSO_4$

It was evident from these results that 850 mg of glucose per 100 c c of the substrate gave proportionately maximum amount of lactic acid with minimum amount of unconverted glucose With this concentration of glucose then various experiments were carried out to see whether the yield of lactic acid could be increased Since inorganic phosphate and Mg ions are known to increase the yield of lactic acid, these were added to the substrate and results are recorded in Table II The final volume of the substrate was always in the proportion of one part veal and two parts water (including all ingredients added)

TABLE II

Substrate containing per 100 g veal, 1,700 mg of glucose and	Final glucose conc in mg per 100 c c	Lactic acid in mg per 100 c c
(1) 50 c c 0 05 M K_2HPO_4 or Na_2HPO_4	52	500
(2) 75 c c 0 05 M K_2HPO_4 or Na_2HPO_4	100	428
(3) 100 c c 0 05 M K_2HPO_4 or Na_2HPO_4	190	236
(4) Containing 50 c c 0 05 M phos solution *plus* 10 mg Mg as $MgCl_2$	160	395
(5) Containing 50 c c 0 05 M phos solution *plus* 20 mg Mg as $MgCl_2$	163	390

As the substrate containing 50 c c 0 05 M phosphate solution gave good results Mg ion in the form of $MgCl_2$ was added to the substrate and results as noted above are recorded

Since the yield of lactic acid fell far short of the theoretical amount ($C_6H_{12}O_6 = 2\ CH_3\ CHOH\ COOH$ or 180 g glucose would give 2×90 g of lactic acid), it was thought that either the duration of the process was too long or too short in the first case a reversible reaction sets in re-converting lactic acid into its parent substance or it is decomposed by micro-organisms and in the second case full conversion was not taking place and other intermediate compounds are being formed Accordingly the following experiments were carried out —

(1) Following the course of the process every 3 hours (2) Carrying the process at 20°C, instead of at 30°C (3) Addition of toluene to stop bacterial growth (4) Search for the intermediate products such as pyruvic acid glyceraldehyde, etc which are known to be formed during glycolysis

TABLE III

Substrate with 1,700 *mg glucose* plus 50 *c c of* 0 05 *M phosphate per* 100 *g veal*

	Final glucose in mg per 100 c c	Lactic acid in mg per 100 c c
(1) Course of the process —		
3 hours	364	200
6 ,,	260	240
9 ,,	159	340
12 ,,	50	500

TABLE III—*concld*

	Final glucose in mg per 100 c c	Lactic acid in mg per 100 c c
(2) Carrying the process at 20 C —		
15 hours	400	200
30	300	250
(3) Addition of toluene at 30 C —		
12 hours	270	200

A search for pyruvic acid glyceraldehyde and other carbonyl compounds which combine with bi-sulphite and which are known to be formed during glycolysis, was made according to the method of Clift and Cook (1932) and Klein (1940) and in almost all cases these were found to be absent Methylglyoxal was estimated according to the method of Baner and Ziegler (1937), but this was also absent

It has been observed during a large number of experiments that a few samples of veal did not give satisfactory conversion of glucose even after the addition of phosphate, and it was thought desirable in any routine procedure to test the sample of veal as regards the potency of the enzyme present in it A sample test was done with a substrate containing phosphate and 200 mg to 250 mg of added glucose A good sample would convert 100 mg to 120 mg of glucose in one hour at 30°C If any sample did not show this conversion, the sample was rejected It may be mentioned here that in such substrate generally some carbonyl compounds of bi-sulphite combining capacity are found showing that the conversion was not complete

Discussion

Various theories as regards the mode of action, the nature of co-enzyme intermediate products formed, etc , in the glycolytic process have been advanced Summing up these observations, it may be taken as certain that phosphate ester of glucose is formed as an intermediate product and a co-enzyme is necessary for the intermediate phosphorylation and de-phosphorylation—the higher the degree of phosphorylation, the greater the amount of conversion Co-enzyme was not separately added as it is present in meat extract but inorganic phosphate was added to the substrate with the idea that it would increase the degree of phosphorylation and it does so up to a certain concentration after which inhibition of the process takes place (*vide* Table II) It has been shown by Cori and Cori (1937) that Mg ion greatly increases the conversion of the glucose ester—the optimum concentration being 5 mM to 10 mM Experimental results in Table II showed a fall in the degree of conversion of glucose This may be explained by the fact that the average Mg content of muscle is about 10 mM as shown by Schmidt and Greenberg (1935) and a fall is due to the sub-optimal concentration of Mg It is only in dialysed extract Mg shows a beneficial effect

Addition of toluene to prevent bacterial decomposition did not show any improvement, on the other hand an inhibitory effect was noticed Neuberg (1929) has recently shown that the enzyme is partly or wholly lost in presence of toluene The lower yield of lactic acid than the theoretical amount can only be explained on the basis of the observation of Colowick *et al* (1940) that glucose though it serves as an oxidizable substance in glycolytic process, is not quantitatively converted into lactic acid and other C_1 and C_2 compounds are formed From the results given in the text it appears that the whole process, as carried out under the conditions of experiments, is mainly the combination of two types of glycolysis animal glycolysis type and fermentation type in varying degree

Sarcolactic acid thus produced has been purified by the preparation of its zinc salt and the optical activity of the pure acid thus prepared has always been verified For the utilization of the lactic acid, it may be pointed out that Mueller (1938) has shown that utilization of lactic acid by *C diphtheria* is pretty definitely limited to the d-form occurring naturally in body tissue Various workers have used lactic acid in the form of ammonium or sodium salt for the production of diphtheria toxin but they have not mentioned the optical rotation of the acid they have used The author intends to take up a work in future with d- and l-form lactic acid and to study their effect on the diphtheria toxin production

Summary

A rapid and inexpensive method for the production of sarcolactic acid, by the glycolytic conversion of glucose in muscle infusion, is described

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Ind Jour Med Res, 31, 2, October, 1943

THE EFFECT OF STORAGE ON THE CAROTENE CONTENT OF DEHYDRATED VEGETABLES

BY

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[Received for publication, April 22, 1943]

In a previous paper (Sekhon, 1942), an account was given of the effect of dehydration and reconstitution on the carotene content of various dehydrated vegetables Some preliminary tests on the effect of storage were carried out, these indicated that the stability of carotene on storage varied considerably in different vegetables Since dehydrated vegetables are normally stored for many months before consumption, it was thought advisable to carry out further work on losses of carotene on storage Ranganathan (1942) has shown that destruction of vitamin C in dehydrated vegetables during storage for 1 to 4 months is fairly rapid

The investigation covered a period of storage of about 20 weeks, the vegetables being kept under varying conditions It was found that the samples of cabbage, cauliflower, carrot and pumpkin kept in sealed tins in an incubator darkened in colour and developed an offensive smell after about 4 months' storage The samples for test were kindly supplied by Messrs Parry & Co, Ltd, Rampet

Experimental

Method of carotene assay—The 'phase separation' test described by Palmer (1922) and subsequently employed by De (1936) was used for estimating carotene The details of the procedure have already been described by Sekhon (*loc cit*) Owing to the non-availability of petroleum ether, a solvent supplied by the Burmah-Shell Oil Storage and Distributing Co of India Ltd, Madras, was used in its place

The results are expressed as micrograms of carotene per 100 g of dehydrated (not moisture-free) vegetable as received from the manufacturers One microgram may be taken as approximately equivalent to 1 International unit of vitamin A (De, 1936*a*)

Six vegetables—bitter gourd, cabbage, cauliflower, carrot, potato and pumpkin—were obtained for the investigation Ordinary methods of dehydration were followed All the samples were 'scalded' by being dipped in boiling 0 8 per cent sodium sulphite solution before dehydration (Sekhon, *loc cit*) The samples of the various vegetables were obtained from a single day's batch, and the investigations started within a couple of days of their manufacture

Four sealed tins of each vegetable were received One sealed tin of each vegetable was opened and the sample immediately assayed for carotene From the same tin samples were weighed out and wrapped in paper as soon as the tin was opened These samples were kept in unsealed containers The remaining sealed tins were placed in an incubator at 37°C and taken for test at intervals of about 4 weeks The paper packets containing weighed samples from the first tin were placed in closed but not sealed tins to cut off light and access of air These tins were stored at room temperature (18°C to 23°C) Another series of paper packets containing the weighed samples were put in glass-bottles exposed to diffused light and kept at room temperature The moisture content of the samples kept in paper packets was not determined when later estimations of carotene were carried out since they had been weighed at the outset of the investigation

Results

The results are given in Table I It is to be observed that losses in carotene content on storage are quite considerable, being very rapid during the first 4 weeks Broadly speaking, about one-half of the total loss observed during the 20 weeks took place during the first 4 weeks Losses in the case of cabbage were high and this observation may hold good for all leafy vegetables

TABLE I

The loss of carotene in dehydrated vegetables stored under various conditions

Vegetable	Condition of storage	Storage in days	Carotene µg/100 g	Percentage loss of carotene
1 Bitter gourd (*Momordica charantia*)	Stored at 37°C in sealed containers	0	2,600	
		28	2,143	17 5
		86	1,992	23 3
		142	1,601	38 4
	Stored at 18°C to 23°C in unsealed containers	0	2,600	
		16	2,370	8 8
		30	2,197	15 5
		96	2,005	22 9
		141	1,642	36 8
	Stored at 18°C to 23°C in glass bottles exposed to diffused light	0	2,600	
		16	1,934	25 6
		29	1,809	30 4
		89	1,685	35 2
		135	1,441	44 5
2 Cabbage (*Brassica oleracea capitata*)	Stored at 37°C in sealed containers	0	1 275	
		32	691	45 8
		80	358	71 9
		138	205	83 9
	Stored at 18°C to 23°C in unsealed containers	0	1,275	
		13	750	41 1
		31	296	76 7
		90	250	80 3
		135	231	81 8
	Stored at 18°C to 23°C in glass bottles exposed to diffused light	0	1,275	
		13	419	67 1
		30	279	78 0
		86	167	86 9
		124	54	95 7
3 Cauliflower (*Brassica oleracea botrytes*)	Stored at 37°C in sealed containers	0	475	
		31	375	21 0
		78	270	43 1
		136	230	51 5
	Stored at 18 C to 23°C in unsealed containers	0	475	
		15	423	10 9
		29	386	18 7
		86	265	44 2
		133	241	49 2
	Stored at 18 C to 23 C in glass bottles exposed to diffused light	0	475	
		15	359	24 4
		29	258	45 6
		84	153	67 7
		127	131	72 4

TABLE I—*concld*

Vegetable	Condition of storage	Storage in days	Carotene µg/100 g	Percentage loss of carotene
4 Carrot (*Daucus carota*)	Stored at 37°C in sealed containers	0	75,630	
		27	48,370	36 0
		83	44 215	41 5
		143	28,040	62 9
	Stored at 18°C to 23°C in unsealed containers	0	75,630	
		17	68 190	9 8
		29	58,800	22 2
		92	43,050	43 0
		141	28,260	62 6
	Stored at 18°C to 23°C in glass bottles exposed to diffused light	0	75,630	
		16	68,390	9 5
		28	54,460	28 0
		88	36,340	51 9
		132	26 880	64 4
5 Pumpkin (*Cucurbita maxima*)	Stored at 37°C in sealed containers	0	30,330	
		69	24,000	20 8
		128	22,380	26 2
		143	18,274	39 6
	Stored at 18°C to 23°C in unsealed containers	0	30,330	
		27	26,000	14 2
		93	20,250	33 2
		140	15,180	49 9
	Stored at 18°C to 23°C in glass bottles exposed to diffused light	0	30 330	
		16	27 840	8 2
		28	25,260	16 7
		89	19,170	36 8
		132	15,650	48 7
6 Potato (*Solanum tuberosum*)	Stored at 37°C in sealed containers	0	82	
		84	64	21 9
		143	54	34 1
	Stored at 18°C to 23°C in unsealed containers	0	82	
		17	79	3 6
		38	72	12 2
		96	66	19 5
		142	58	29 2
	Stored at 18°C to 23°C in glass bottles exposed to diffused light	0	82	
		31	71	13 4
		90	64	21 9
		135	56	31 7

The effect of temperature—Within the observed range (18°C to 37°C) temperature did not appear to have a striking influence on the stability of carotene

The effect of light—Losses in samples in the glass-bottles exposed to light were more than those which took place in the samples not exposed to light (Guilbert, 1935) This effect appeared to be most marked in samples containing a high percentage of chlorophyll In the present investigation losses in cabbage were of a much higher order than those observed in the other samples In the case of carrot, pumpkin and potato the effect of exposure to light was not marked

Some preliminary observations were made on a number of samples of dehydrated vegetables prepared by the 'quick-cooking' process, in which the vegetables are steamed for about 15 minutes before dehydration Subsequently the material was made into briquettes under pressure Samples for analysis were taken from the inside of the briquettes The results are given in Table II —

TABLE II

The loss of carotene in dehydrated vegetables prepared by the 'quick-cooking' process, stored at 37°C *

Vegetable	Period of storage (days)	Carotene µg /100 g	Percentage loss of carotene
Cabbage	0	308	
	36	217	29 5
Cauliflower	0	341	
	36	297	12 9
Pumpkin	0	5,932	
	36	5,238	11 7
Turnip	0	25	
	36	25	*Nil*

* These samples were sent to the Laboratories some days after their manufacture

The results obtained in this case corresponded in general with those of the samples prepared in the ordinary way, suggesting that this form of preliminary treatment did not affect the stability of carotene in dehydrated vegetables However, during the process of 'quick-cooking' carotene appears to be rapidly destroyed, as judged by the figures obtained for carotene for different fresh vegetables by the author

SUMMARY

1 The effect of storage on the carotene content of six dehydrated vegetables has been studied

2 The dehydrated vegetables on storage showed a progressive loss in their carotene content

3 Temperature, within the observed range (18°C to 37°C), appeared to have little influence on the rate of destruction of carotene in dehydrated vegetables

4 The destruction of carotene in vegetables containing a high percentage of chlorophyll was more pronounced in the presence of light

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COMBINED ESTIMATION OF THIAMIN AND NICOTINIC ACID IN FOODSTUFFS BY CHEMICAL METHODS

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[Received for publication, May 5, 1943]

In recent years, numerous chemical methods have been described for the individual estimation of thiamin and nicotinic acid In the present investigation, a simple procedure is evolved whereby a combined estimation of thiamin and nicotinic acid could be made on the same sample Further, this procedure enabled the assay of 5 to 6 foodstuffs in one day

The chemical methods employed for the estimation of thiamin fall into two groups (1) colorimetric methods (Prebluda and McCollum, 1939, Melnick and Field, 1939, Kirch and Bergeim 1942), and (2) the thiochrome method, first described by Jansen (1936) and later modified by other workers (Hennessy and Cerecedo 1939, Pyke, 1939, Aykroyd *et al*, 1940, Housten *et al*, 1940, Harris and Wang, 1941, Swaminathan, 1942) with a view to applying it to foodstuffs

The following steps are involved in the estimation of thiamin by the thiochrome method —

1 *Extraction of thiamin from foodstuffs* —Preliminary hydrolysis to liberate the vitamin from its biological combinations presumably with proteins, has been carried out either with hydrochloric (Aykroyd *et al loc cit*) or sulphuric (Conner and Straub, 1941, Swaminathan, 1942) or acetic acids (Hennessy and Cerecedo, *loc cit*) or with pepsin (Pyke, *loc cit*), or papain (Harris and Wang *loc cit*) The extraction with acid is not satisfactory, since (*a*) acids in general will extract, besides thiamin, other extraneous substances and (*b*) large amounts of the solvent are necessary to obtain anything like complete extraction Enzymic hydrolysis may prove to be the best means of liberating and extracting the vitamin

2 *Conversion of cocarboxylase to thiamin* —Lohman and Schuster (1937) have shown that the greater part of naturally occurring thiamin may be present as cocarboxylase (i e phosphorylated thiamin) Cocarboxylase is converted by alkaline ferricyanide to the corresponding thiochrome, which Kinnersley and Peters (1938) have shown to be insoluble in isobutyl alcohol, thus its presence might be a source of error in the chemical test for thiamin Hence, in any method for the estimation of thiamin, a conversion of cocarboxylase to thiamin is essential and this is achieved by subjecting the acid or enzymic hydrolysates to the action of a phosphatase Phosphatases from various sources can be used Thus, Hennessy and Cerecedo (*loc cit*) used a preparation of beef kidney, which, however, was found to be unsatisfactory by Melnick and Field (1939) who recommended dried yeast as a source of phosphatase Kinnersley and Peters (*loc cit*) used taka diastase, which contained a taka phosphatase In the case of cereals and pulses, however, preliminary digestion with a phosphatase is unnecessary, as the vitamin in these is almost entirely present in the free form In the present investigation, enzyme preparation prepared from pig's intestinal mucosa was used This contained proteases as well as phosphatases, the liberation and conversion of cocarboxylase to thiamin being thus achieved in a single operation

3 *Removal of interfering substances* —In order to get rid of the substances interfering with the reaction. Karrer and Kubli (1937) and Hennessy and Cerecedo (*loc cit*) recommended the use of zeolite and Decalso for the adsorption of thiamin Adsorption methods, apart from being tedious, involve a risk of incomplete adsorption and elution, hence, if possible, they should be avoided Harris and Wang (*loc cit*) and Swaminathan (1942) suggested extracting the solution with isobutyl alcohol prior to oxidation to thiochrome In the present experiments, however, preliminary washing with isoamyl alcohol prior to oxidation was found to be unnecessary with all the foodstuffs except yeast extracts, as the treatment with basic lead acetate effectively removed interfering substances, as judged by the low blanks obtained in every case

4 *Oxidation of thiamin to thiochrome and the amount of ferricyanide required for the oxidation*—Thiamin is converted to thiochrome by alkaline ferricyanide The amount of potassium ferricyanide used influences the oxidation Several workers (Hills, 1939, Aykroyd *et al*, *loc cit*, Jowett, 1939, Westenbrink and Goudsmit, 1937) have shown that excess of ferricyanide destroyed part of the thiochrome In view of these observations, the effect of various concentrations of ferricyanide was tried with a brewer s yeast hydrolysate Relevant results are given in Table I —

TABLE I

The effect of different concentrations of ferricyanide on the intensity of fluorescence

Amount of ferricyanide in mg	Intensity of fluorescence (in scale divisions)
0 0	8 5
1 0	40 0
2 0	51 0
4 0	52 0
6 0	52 0
8 0	51 0
10 0	48 0
12 0	47 0
15 0	47 0
18 0	47 0
20 0	47 0
24 0	47 0
30 0	47 0

Thus, from 2 mg to 8 mg of ferricyanide was found to be enough for the oxidation of thiamin to thiochrome In all subsequent tests 0 3 ml (6 mg) of 2 per cent ferricyanide solution was used

5 *Extraction of thiochrome*—Isobutyl alcohol is generally used for the extraction of thiochrome from aqueous solutions Owing to the unavailability of this solvent, a substitute isoamyl alcohol, which was found to be an equally good solvent for thiochrome (Narasinga Rao, 1943), was used in the present investigation

6 *Measurement of the intensity of fluorescence*—Thiochrome shows an intense blue fluorescence in the ultra-violet light, the intensity of which is measured by means of a photo-electric fluorimeter

For the estimation of nicotinic acid in biological materials, a colorimetric method first described by Swaminathan (1938, 1938*a*) is much in vogue Numerous modifications (Shaw and Macdonald 1938 Pearson, 1939, Ritsert, 1939, Kringstad and Naess, 1939, Harris and Raymond, 1939 Bandier and Hald, 1939 Melnick and Field, 1940, Kodicek, 1941, Swaminathan, 1942) have been proposed based on the mode of extraction and the choice of aromatic amine used in the reaction All the procedures described for the extraction of nicotinic acid are, however, drastic and give rise to highly-coloured solutions especially with materials of plant origin, thereby necessitating separate blank estimations in every case Melnick and Field (1940), Giri and Naganna (1941) and Dann and Handler (1941) described adsorption methods for the separation of nicotinic acid from interfering substances, thereby minimizing the blanks Melnick and Field (1941) have also emphasized the proper evaluation of the blank, otherwise considerable error was introduced in the calculations They recommended omission of aniline from the blank test as they found aniline to react directly with certain substances present in hydrolysate to yield colours indistinguishable from nicotinic acid Bandier (1939) on the other hand advocates the inclusion of cyanogen bromide in the blank, which, however, has been found to be unsatisfactory Kodicek (*loc cit*) and Waisman and Elvehjem (1941), by direct extraction with alkali or acid obtained higher values for the nicotinic acid content of cereals than could be accounted for by their biological potency In the present study, enzymic hydrolysis was employed for the liberation and extraction of nicotinic acid from combined

forms such as coenzymes I and II. The final solutions obtained with the majority of foodstuffs were practically colourless or contained only traces of yellow which were so small that they could not be estimated by an ordinary Klett colorimeter.

Experimental

Reagents required —

1. M/10 phosphate buffer pH 6 to 7
2. Sodium acetate solutions, 50 per cent and 5 per cent
3. N basic lead acetate solution
4. 10 N H_2SO_4
5. 10 N NaOH
6. Standard thiamin solution prepared daily by diluting 1 ml of stock thiamin solution to 100 ml thus 1 ml = 1 μg thiamin
7. Distilled methyl alcohol
8. Distilled isoamyl alcohol
9. Two per cent potassium ferricyanide solution prepared fresh as required
10. Standard nicotinic acid solution prepared daily by diluting 1 ml of stock nicotinic acid solution to 10 ml thus 1 ml = 50 μg nicotinic acid
11. Two per cent aniline solution
12. Cyanogen bromide solution prepared as required by decolorizing ice-cold saturated bromine water by addition of 10 per cent NaCN solution

Preparation of the enzyme —The enzyme preparation was prepared from the intestinal mucosa of a healthy starved pig (Pig's intestinal mucosa was chosen, since it is known to contain a large and varied array of enzymes and it is easily obtainable). A thick, viscous suspension of the mucosa was collected to which an equal volume of 1·0 per cent saline was added and the mixture was centrifuged. The supernatant was discarded, the residue was suspended in an equal volume of 1 per cent saline and preserved in the ice-box over toluene. The activity of the preparation was found to keep for months. It contained proteases, phosphatases, nucleotidases and other enzymes. No attempt was made to purify the preparation, since (*a*) the object was merely to liberate and extract the two vitamins from their biological combinations and (*b*) the preparation was found to be completely devoid of thiamin and nicotinic acid as determined by the chemical methods (Table II).

Table II

Thiamin and nicotinic acid in the enzyme preparation

Enzyme preparation	Thiamin		Nicotinic acid	
	μg/g	Percentage recovery of added thiamin	μg/g	Percentage recovery of added nicotinic acid
I	Nil	100·0	Trace	100·5
II	Nil	100·0	Nil	100·0
III	Nil	100·0	Nil	100·0

Preliminary experiments

Effect of pH on the liberation and extraction of thiamin and nicotinic acid —In order to find the optimum pH for hydrolysis with the mucosa-enzyme preparation, a sample of brewer's yeast was used. Acetate buffer was used to obtain pH from 4 to 5·65, while pH 7·0 was obtained by addition of 5 per cent sodium-acetate solution. For comparison hydrolysis at

pH 7 0 was carried out in the presence of phosphate buffer The experimental details were as outlined below The results are presented in Table III —

TABLE III

Effect of pH on the liberation and extraction of thiamin and nicotinic acid

pH	THIAMIN		NICOTINIC ACID
	µg /g	Percentage recovery of added thiamin	µg /g
4 0	100 5	90 9	186 8
5 0	100 5	90 9	359 0
5 6	97 9	92 3	436 7
7 0	100 5	90 9	443 0
7 0 (Phosphate buffer)	100 5	90 9	440 0

It will be seen from Table III that thiamin could be extracted over a wide range of pH varying from 4 to 7 Further, there was practically no destruction of thiamin as judged from the percentage recovery of the added vitamin, when the hydrolysis was carried out at pH 7 0 for 24 hours In the case of nicotinic acid, however, the optimum pH for the enzyme—presumably nucleotidase to liberate nicotinamide, since nicotinic acid exists in nature almost entirely as free and combined nicotinamide (Euler *et al* , 1937)—appears to be nearer pH 7 0 Thus, in all subsequent experiments, the hydrolysis with the mucosa preparation was carried out at pH 6 to 7, with either phosphate or acetate buffers

WORKING DIRECTIONS

Extraction of thiamin and nicotinic acid —From 1 g to 10 g of the finely powdered materials were well mixed with 50 ml to 100 ml of phosphate buffer pH 6 to 7 or with water and the pH was adjusted to 6 to 7 by the addition of 5 per cent sodium-acetate solution One ml of the enzyme preparation was added and the mixture was incubated overnight at 37°C Toluene was added as a preservative A parallel incubation was carried out with a further sample of the same material, to which 50 µg of thiamin and 300 µg of nicotinic acid were added The mixture was centrifuged, the residue was washed once with phosphate buffer or sodium-acetate solution as the case may be The centrifugates were mixed and made up to 50 to 100 ml

Removal of interfering substances —To 50 ml of the centrifugate, 10 ml of N basic lead acetate were added in order to precipitate interfering substances The solution was centrifuged and the precipitate was discarded The excess of lead was removed as lead sulphate by the addition of 2 ml of 10 N H_2SO_4 The precipitate was removed on the centrifuge and the supernatant was brought to pH 4 0 by the addition of 10 N NaOH (solution A) In all cases, clear, practically colourless, solutions were obtained

Estimation of thiamin —For the estimation of thiamin, an aliquot (usually 6 5 ml) of solution A, corresponding to 1/10 of the material, was used It was made up to 13 ml with water , 2 ml methyl alcohol 2 ml 10 N NaOH, 0 3 ml 2 per cent ferricyanide solution and 15 ml isoamyl alcohol were added and the mixture was shaken for 2 minutes When the amyl alcohol had separated from the aqueous solutions, it was filtered and 10 ml used for the fluorimetric estimation of thiamin A standard thiamin solution (5 µg) treated in the same way was used for comparison For each test solution a corresponding blank estimation was done All the values obtained with test materials (after deducting the values obtained with their corresponding blanks) were corrected using the corresponding recovery values

Estimation of nicotinic acid —Another portion (20 ml to 50 ml) of solution A was taken for the estimation of nicotinic acid It was heated in a boiling water-bath for 30 to 40 minutes in order to hydrolyse the nicotinic amide formed to nicotinic acid During hydrolysis, considerable

evaporation of the solutions took place The solutions were brought to pH 7 0 and made up to their original volume in the case of yeast yeast extracts and other nicotinic-acid-rich materials, while in the case of cereals and pulses, the volume was made up to half the original volume and filtered An aliquot of the solution corresponding to 0 1 (yeast)—2 g (cereals and pulses) was used for the estimation of nicotinic acid by Swaminathan's method (1942)

The thiamin and nicotinic acid contents of different foodstuffs analysed are given in Table IV —

TABLE IV

Thiamin and nicotinic acid content of foodstuffs with percentage recovery of added vitamins

Name of foodstuff		Amount taken, g	Thiamin µg /g	Thiamin Percentage recovery of added thiamin	Nicotinic acid µg /g	Nicotinic acid Percentage recovery of added nicotinic acid
Yeasts						
Brewer s	(1)	1	70 8	80 0	322 6	
	(2)	1	70 8	87 0		
, (sun dried)	(3)	1	97 9	92 3	436 7	95 0
	(4)	1	86 1	90 0	465 0	95 0
Torula (grown on molas es)	(1)	2	19 7	79 2	120 1	100 0
,,	(2)	2	22 1	74 4	120 0	101 0
,, (sun dried)	(3)	2	26 0	97 8	215 6	93 0
Distillery	(1)	2	10 1	78 0	244 3	100 0
,,	(2)	2	15 8	75 0	322 5	97 0
Yeast extract	(1)		15 5	83 9	285 0	107 0
,,	(2)				470 8	90 0
Nuts [Ground nuts (*Arachis hypogea*)]						
Raw		5	8 2	89 6	33 0	105 0
Ether extracted	(1)	2 5	16 7	99 0	73 5	100 0
,	(2)	2 5			79 4	102 0
Flesh foods						
Sheep liver	(1)	5	5 8	88 6	168 5	100 0
,,	(2)	5	2 8	90 0	107 0	100 0
Sheep heart		5	4 8	95 2	35 1	
Pulses						
Bengal gram *a*	(1)	10	4 9	87 0	20 0	93 3
,,	(2)	10	4 4	86 0		
Red gram *b*		10	3 8	102 0	23 6	86 0
Horse gram *c*	(1)	10	5 3	85 7	20 4	90 0
,	(2)	10	5 0	86 7	23 5	90 0
Cow pea *d*	(1)	5	8 3	60 0	17 7	97 5
,	(2)	5	8 4	64 0		
Cereals						
Wheat *e*	(1)	10	3 6	79 0	23 3	100 0
,	(2)	10	3 9	89 0	23 8	96 0
White maize *f*		10	4 2	101 1	11 8	105 0
Bajra *g*	(1)	10	3 0	57 7	11 3	
,, (ether extracted)	(2)	10	2 7	57 7	13 8	83 0
,,	(3)	10	2 8	57 7	16 2	
Rice, milled *h*		10	1 04	80 0	14 1	100 1
Rice polishings		3	18 1	46 0	85 5	93 3
Ragi *i*	(1)	10	3 3	30 0		
,,	(2)	10	4 2	20 0		
,,	(3)	10	3 8	20 0		
Miscellaneous						
Wheat biscuits		20	1 28	73 8	26 7	99 7
Pumpkin seeds *j* (ether extracted)		5	3 8	80 0	19 0	100 0

A B —*a*=*Cicer arietinum*, *b*=*Cajanus indicus* *c*=*Dolichos biflorus*, *d*=*Vigna catiang* *e*=*Triticum vulgare*, *f*=*Zea mays*, *g*=*Pennisetum typhoideum* *h*=*Oryza sativa*, *i*=*Eleusine coracana* *j*=*Cucurbita maxima*

For four samples of brewer's yeast the values for thiamin were 70 8 μg /g to 97 9 μg /g They were very much higher than those reported by Swaminathan (1942) The thiamin content of torula yeast was found to be lower than that of brewer s yeast while distillery yeast gave the lowest values This is in agreement with known facts Nicotinic acid values in general, were of the same order as observed by different workers Samples of yeast, which were dried in the sun, gave higher values for thiamin and nicotinic acid than those dried otherwise A marked variation in the thiamin and nicotinic acid content of sheep liver has been observed and this may be due to the variation in age and nutritive condition of the animal as indicated by Waisman *et al* (1940) The four pulses investigated were found to be good sources of thiamin , cow-pea giving a value of 8 4 μg /g was the richest amongst them The nicotinic acid content of both cereals and pulses were found to be very much lower than that of ground-nuts and animal tissues such as sheep liver Recoveries of added thiamin and nicotinic acid were satisfactory In the case of three cereals bajra, ragi and rice polishings, and one pulse cow-pea the recoveries of added thiamin ranged from 20 to 60 per cent The following preliminary experiments were carried out with ragi and rice polishings to find out the causes for the low recoveries obtained

(*a*) Samples were extracted prior to hydrolysis with ether to see if substances interfering with the reaction could be removed (*b*) the period of hydrolysis was shortened to 3 hours, (*c*) the aqueous suspensions of the materials were heated in a boiling water-bath prior to enzymic hydrolysis , in order to inactivate substances which might be responsible for the destruction of thiamin , 50 μg thiamin were added and the suspension incubated overnight and (*d*) a known amount of thiamin was added to the purified solutions prior to oxidation to see whether the low values obtained were due to the destruction of thiamin by substances present in the hydrolysate The results of the preliminary experiments are given in Table V —

TABLE V

Foodstuff	Treatment	Thiamin		Percentage recovery of thiamin added prior to oxidation
		μg /g	Percentage recovery of thiamin	
Ragi (1)	Ether extraction	3 8	20 0	93 2
(2)	3 hours' hydrolysis	3 8	16 0	95 0
Rice polishings (1)	Ether extraction	18 0	46 0	93 9
, , (2)	3 hours' hydrolysis	18 4	47 4	
,, ,, (3)	Heating in water bath for 1 hour prior to hydrolysis	15 3	71 0	

It appears from Table V that part of the added thiamin was lost when incubated with the suspensions of the two cereals Extraction of the sample with ether or shortening the period of hydrolysis did not seem to minimize the loss of thiamin However, better recovery was obtained with rice polishings, when they were heated in boiling water for some time prior to enzymic hydrolysis This is of interest in view of the findings of Woolley (1941) and Spitzer, Coombes and Elvehjem (1941) that thiamin was inactivated by certain substances present in raw fish The inactivation was reduced when the fish was cooked Whether such substances are present in rice or ragi is a matter for future investigation That lower recoveries were not due to the presence of interfering substances was borne out by the almost complete recoveries obtained when thiamin was added to the purified extracts just before oxidation to thiochrome

Liberation of thiamin and nicotinic acid from foodstuffs on autolysis —In foodstuffs thiamin exists in free and phosphorylated forms and nicotinic acid, almost entirely as free and combined as (coenzymes I and II) nicotinamide (Euler *et al* 1937) Myrback (1927), Euler and his colleagues (1928 1936, 1937 1938) and Schlenk (1940) have shown that disintegration of

animal and plant tissues releases a heat-labile system which rapidly destroys the biological activity of pyridine nucleotides e g cozymase the optimum pH of inactivation being pH 7 5 The activity of this system can be inhibited by nicotinamide (Mann and Quastel, 1941) Further Handler and Klein (1942) have shown that the initial step in the inactivation of pyridine nucleotides is the cleavage of nicotinamide In view of these observations and the universal occurrence of pyridine nucleotides in plants and animals it was felt of interest to see whether enzyme systems responsible for their inactivation were present in foodstuffs Finely powdered materials were suspended in water and the pH was adjusted to 6 to 7 with 5 per cent sodium acetate and the suspensions were allowed to autolyse overnight in presence of toluene at 37°C Thiamin and nicotinic acid were estimated as described above The results are included in Table VI —

TABLE VI

Liberation of thiamin and nicotinic acid from foodstuffs on autolysis

Foodstuff		Thiamin		Nicotinic acid	
		µg g	Percentage recovery of added thiamin	µg g	Percentage recovery of added nicotinic acid
Yeast					
Brewer's (dried on hot tray)		52 0	81 7	322 6	
(sun-dried)		96 2	96 0	436 7	95 0
Torula (grown on molasses) (sun dried)		20 9	104 0		
Yeast extract		16 0	71 4	285 0	
Flesh foods					
Sheep liver		2 9	90 0	107 0	97 5
Sheep heart		3 8	81·0	37 1	
Nuts					
Ground nut (raw)		7 1	115 0	33 0	108 0
(ether extracted)				79 4	102 0
Pulses					
Bengal gram		5 2	83 0	18 6	90 0
Red gram		3 5	96 0	20 0	100 0
Horse gram		5 0	85 7	20 4	90 0
Cow pea		8 3	60 0	17 7	97 5
Cereals					
Wheat		3 9	79 0		
White maize		4 7	86 4	9 8	92 8
Rice polishings		15 3	50 0	83 3	83 0
Bajra	(1)	2 8	62 0	11 3	
,	(2)	2 7	57 7	13 8	83 0
Ragi		3 75	30 0		

The results presented in Table VI compare well with those given in Table IV This indicates that flesh foods, sun-dried yeast preparations, cereals and pulses contained enzymes responsible for the liberation of thiamin and nicotinic acid However, the use of the enzyme preparation is recommended to ensure complete liberation of the vitamins from all foods

SUMMARY

1 A simple, reliable and rapid method has been described, whereby combined estimations of thiamin and nicotinic acid can be made on the same sample This procedure enables the assay of 5 to 6 foods in a 7-hour day

2 Hydrolysis at pH 6 to 7 by an enzyme preparation from pig's intestinal mucosa has been employed for the liberation and extraction of thiamin and nicotinic acid Further,

the method has also been found to be effective in reducing the interference by extraneous substances to a minimum

3 The thiochrome method has been adopted for the estimation of thiamin and the cyanogen-bromide-aniline method for the estimation of nicotinic acid

4 Various foodstuffs have been assayed for their thiamin and nicotinic acid content and the values obtained were in good agreement with those reported by other workers

5 Recoveries of added vitamins were satisfactory, ranging from 75 to 100 per cent for thiamin and 83 to 108 per cent for nicotinic acid

6 In the case of three cereals and one pulse, recoveries of added thiamin ranged from 20 to 60 per cent An explanation has been offered to account for the low recoveries observed

7 Autolysis of an aqueous suspension of flesh foods has been found to liberate thiamin and nicotinic acid from their biological combination However, the use of the enzyme preparation is recommended in order to ensure complete liberation of the vitamins from all types of foods

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Ind Jour Med Res 31, 2 October, 1943

THE EFFECT OF VITAMIN C ON GINGIVAL AND PERIODONTAL DISEASE IN INDIAN CHILDREN

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[Received for publication September 2 1943]

GINGIVITIS and periodontal disease are extremely common in the population of the Punjab This is found in varying stages ranging from slight thickening of the gums in its earlier and milder stages to a later stage of very marked hypertrophy with marginal ulceration and the formation of pus-pockets (pyorrhœa) Among males of middle age the incidence of pyorrhœa is almost one hundred per cent Contrary to experience in Western countries, gingivitis is also common among children and is some times observed in children as young as 5 years of age The incidence of gingivitis and periodontal conditions in the population will be described in a later communication While little is really known about their ætiology, it has often been suggested that their prevalence is due to some defect in the diet and in particular to deficiency of vitamin C The present paper describes an experiment on children in Lahore designed to test the latter hypothesis

GINGIVITIS AND VITAMIN C

That scurvy affects the gums has been recognized from very early times Thus Sir Richard Hawkins in 'Observations on His Voyage to the South Sea', published in 1593, says that the disease 'causeth a general swelling of the gums and many times the teeth fall out of the jaws without pain' In guinea-pigs fed on diets deficient or almost deficient in vitamin C, lesions of the gingival and periodontal tissues have been observed and described by many workers (Howe 1920, 1921, 1921*a*, 1923, Wolbach and Howe, 1924, Hojer and Westin, 1925, Day 1933, Boyle, Bessey and Wolbach 1937, Boyle 1938, 1941) But while the association of gingivitis in human beings and guinea-pigs with severe deprivation of vitamin C leading to signs of scurvy is fully recognized, there is little unanimity as the part played by vitamin-C deficiency in the ætiology of gingivitis not clinically associated with scurvy Crandon, Lund and Dill (1940) Freeman and Glass (1938), Kirkpatrick (1939), Radusch (1939) and Tishler (1929) did not find any significant relationship between vitamin-C intake and the occurrence of gingivitis Burril (1942) found that the blood plasma vitamin-C levels tended to be lower in the cases having gingivitis than in those free from gingivitis The cases with periodontal disease also tended to show lower vitamin-C levels than those without In proportion to the range and variation in vitamin-C levels within each group, however, the differences between groups with and without periodontal disease were very small and probably not significant The seasonal variations in vitamin-C levels and the incidence of gingivitis were such that no causal relationship between low vitamin C and the presence of gingivitis was apparent Fox, Dangerfield, Gottlieb and Jokl (1940) found no significant difference in the incidence of conditions in two groups of mine labourers in South Africa, one of which received a daily ration of concentrated orange juice for seven months Westin (1925), Bunting (1929), Boyle *et al* (1937*a*), Pelzer (1938), Weisberger, Young and Morse (1938) and Fitzsimmons (1941) reported, however, a definite correlation between vitamin-C intake and gingivitis and associated conditions, while Hanke (1930, 1933, 1933*a*) and Hawkins (1931) recorded definite improvement in gingivitis, when large quantities of orange juice were added to the diet

Experimental

One hundred Indian children in an orphanage in Lahore were selected The total number of children in the institution was 140 Children below 5 and above 15 were excluded The incidence of gingival conditions among the children was high The diet of the institution is based on atta (whole wheat or wheat flour of high extraction) with some pulses Intake of fresh fruits and vegetables, which varies according to season, is in general low The children had been resident in the orphanage from the early years of childhood

Two groups of 50 were selected, as similar as possible as regards age, sex weight and the incidence of gum conditions The presence and severity of gingivitis were determined by oral inspection The incidence of plaques and tartar and the state of 'oral hygiene' were also recorded The following classification was used —

	Condition
Gingivitis +	Evident though not marked thickening of the gums, the interdental papillæ being affected in some instances
Gingivitis ++	Marked hypertrophy of the gums with or without minute marginal ulceration, and possibly slight pocket formation
Gingivitis +++	Very marked hypertrophy of the gums with marginal ulceration and pocket or pus formation
Plaques and tartar	These were recorded as + ++ +++ according to the degree of severity
Oral hygiene	The general mouth condition was recorded as 'good' 'fair or 'poor

One group was given 100 mg of ascorbic acid orally per child per day for 100 days, the other group receiving no supplement Both groups received the same diet no change being made in the diet of the orphanage Oral examination of all children was carried out at the beginning of the experiment and after 50 and 100 days Table I shows the incidence of plaques and tartar and the state of oral hygiene in two groups The groups were reasonably comparable as regards these conditions In Table II the incidence of malocclusion according to Angle's classification is shown Malocclusion of teeth may predispose to gingivitis and hence it is of interest to note that the incidence was low in both groups

Table I

Plaques tartar and oral hygiene in test and control group

Classification	Plaques		Tartar		Oral hygiene		
	Test group per cent	Control group, per cent	Test group, per cent	Control group per cent	Condition	Test group per cent	Control group, per cent
Normal	14 3	13 5	32 6	24 6			
+	30 5	33 3	22 4	37 0	Good	28 5	29 6
++	32 6	30 8	20 4	22 2	Fair	46 9	41 9
+++	22 4	22 2	24 5	16 0	Poor	24 5	28 4

TABLE II

Incidence of malocclusion (Angle's classification)

Classification	Test group per cent	Control group, per cent
Class I	26·3	19·7
Class II	20·4	13·5
Class III	Nil	Nil
Normal	53·0	66·6

The children were weighed and measured at the beginning and end of the experimental period. All were examined to discover whether any were suffering from certain deficiency diseases—angular stomatitis, phrynoderma, xerophthalmia and rickets—but none of these conditions was observed.

RESULTS

Tables III and IV show that the administration of 100 mg. of ascorbic acid for 100 days did not produce improvement in the gingival conditions present. The lack of effect was evident after 50 days, when it was decided to give oral treatment to half the children in each group. Treatment included extractions, fillings, scaling and gum dressing. After the establishment of a satisfactory state of oral hygiene no further treatment was given. The test group continued to receive ascorbic acid throughout the period of oral treatment. There was some indication that this treatment somewhat improved the gum conditions, but no very striking effect was evident during the experimental period.

Owing to the smallness of the numbers in the experimental and control groups and the uncertainty as to the correctness of the recorded figures for age, weight and height increments in the two groups could not be compared on a satisfactory statistical basis. Rough comparison of the increments in the two groups gave, however, no indication of any advantage in the group receiving ascorbic acid. It may also be added that the giving of the ascorbic acid tablets had no visible and obvious effect on the appearance and health of the children.

EXCRETION OF ASCORBIC ACID

The excretion of ascorbic acid by 20 children in each group was determined during the course of the experiment, the usual method involving the use of 2,6 dichlorophenol indophenol being employed. The children were given bottles containing acetic acid into which urine was passed, the concentration of acetic acid in the urine being about 5 per cent. The bottles were collected twice daily and the urine titrated as soon as possible. The total daily quantity of urine passed into the bottles ranged from 800 c.c. to 1,200 c.c. It was assumed that this represented a 24-hour specimen, but it was difficult to be certain on this point. The tests were carried out during the hottest season of the year in Lahore when urine tends to be concentrated. The shade temperature was frequently from 100°F. to 110°F. Since the method of collection involved storage of some of the urine for a period somewhat exceeding 12 hours before titration, it is likely that some destruction of ascorbic acid took place.

TABLE III

The incidence of gingivitis in the test group receiving ascorbic acid

CHILDREN RECEIVING DENTAL TREATMENT						CHILDREN NOT RECEIVING DENTAL TREATMENT											
Number	Age	Sex	Examination			Number	Age	Sex	Examination			Number	Age	Sex	Examination			Number	Age	Sex	Examination		
			1st	2nd	3rd				1st	2nd	3rd				1st	2nd	3rd				1st	2nd	3rd
1	5	F	+++	++	++	14	11	M	+++	+++	+++	1	5	F	++	+++	+++	13	10	M	+++	+++	+++
2	5	F	+++	+++	+++	15	12	M	++	++	++	2	5	M	+	+	+	14	10	M	+	+	–
3	5	M	N	+	+	16	12	M	+	+	+	3	6	M	++	++	++	15	11	M	+	+	–
4	6	F	+	N	N	17	13	M	+++	+++	+++	4	7	M	N	N	N	16	12	M	N	N	N
5	7	F	++	++	++	18	13	M	+	+	+	5	7	F	++	++	++	17	13	F	+±	++	–
6	8	M	+++	+++	+++	19	14	M	+++	++	++	6	8	M	++	+++	+++	18	13	M	+++	+++	+–
7	8	F	+	+	+	20	14	M	±	±	N	7	8	M	N	N	N	19	13	M	+	N	N
8	9	M	+++	+++	+	21	14	M	++	++	+	8	9	M	+	++	+	20	13	M	++	++	+–
9	9	M	++	+	++	22	14	M	+++	++	+±	9	9	M	+	N	N	21	13	M	++	+	+–
10	9	F	±	+	N	23	15	M	++	++	+	10	9	F	++	++	++	22	14	M	++	+	+++
11	10	M	+	+	+	24	15	M	+	N	N	11	9	F	++	+++	++	23	14	M	+	N	N
12	10	M	++	++	±	25	15	M	++	++	+	12	10	M	+	+	++	24	15	M	+	+	–
13	11	M	+	+	±																		

TABLE IV

Incidence of gingivitis in the control group not receiving ascorbic acid

CHILDREN RECEIVING DENTAL TREATMENT										CHILDREN NOT RECEIVING DENTAL TREATMENT									
Number	Age	Sex	Examination			Number	Age	Sex	Examination			Number	Age	Sex	Examination			Number	Age	Sex	Examination		
			1st	2nd	3rd				1st	2nd	3rd				1st	2nd	3rd				1st	2nd	3rd
1	5	F	+++	+++	++	14	11	M	++	+	\	1	5	I	[illegible]			14	11	M	[illegible]		
2	5	M	++	+	+	15	11	M	+	+	\	2	5	I				15	12	M	\	\	\
3	6	F	++	+	+	16	11	M	+	+	N	3	7	I	-		±	16	13	M	-		- -
4	7	M	+++	+++	+	17	11	F	++	++	+	4	8	M	-	-	-	17	13	M		\	\
5	8	M	+	+	+	18	12	M	N	\	\	5	8	M	+ - -	+ +		18	13	M		-	- -
6	8	M	++	+	N	19	12	M	+	+	+	6	9	M	+	++	+	19	14	M	\	\	±
7	9	F	±	±	+	20	13	M	N	±	\	7	9	M	±	±	+	20	14	M	-	-	- - +
8	9	M	+	+	N	21	13	M	+++	+++	+++	8	9	M	++	+	+	21	14	M	+	- -	-
9	9	M	++	++	++	22	14	M	N	N	N	9	9	M	+++	+++	\	22	14	M	-	++	+
10	10	M	+	+	±	23	14	M	++	+	N	10	10	M	++	++	+	23	14	M	++	+	- -
11	10	M	+++	+++	++	24	14	M	+++	+++	+++	11	10	M	++	+	+	24	15	M	\	+	+
12	10	M	++	+	+	25	15	M	++	++	++	12	11	F	+	++	+++	25	15	M	-	N	N
13	11	F	+++	++	++							13	11	M	++	++	++						

The results are shown in Table V The excretion of ascorbic acid by the control group was very low, while the group receiving ascorbic acid excreted from 25 1 mg to 58 4 mg in 24 hours Subject to the limitations referred to above, the results of the excretion tests suggest that the diet of the control group was low in ascorbic acid

TABLE V

Urinary excretion of ascorbic acid per day per child

TEST GROUP		CONTROL GROUP	
Name	Excretion per day (mg)	Name	Excretion per day (mg)
Parshotam	28 6	Inder Parkas	1 0
Chabir Singh	27 4	Nanu Ram	1 7
Girdhari Lal	35 0	Jai Dayal	2 4
Om Parkash No IV	37 8	Dhian Singh	1 0
Gian Chand No III	43 9	Sukh Nandan	1 2
Budh Dev	44 4	Sham Lal	1 5
Ram Dayal	33 0	Narian Das	1 0
Ram Chand	41 1	Rath Ram	1 8
Om Parkash	53 1	Lakshman	1 8
Durga Charan	36 6	Mohan Lal	2 2
Ram Suroop	58 4	Ram Chand	1 2
Harhans Lal	28 1	Suraj Parkash	1 5
Vishwa Mittra	40 3	Vishnu Prashad	1 0
Brahm Datta	29 1	Jagdish	1 5
Shiv Dayal	44 2	Rameshwar	33 9*
Rattan Lal	29 7	Mansa Ram	1 2
Prem Chand	25 1	Ganga Ram	1 6
Ram Dass	53 9	Sutre Ram	1 2
Krishan Swarup	37 3	Prabh Dayal	2 9
Lal Chand	45 7	Ram Lal	1 5
Roshan Lal	33 4	Sutri Path	1 5

* Took two pounds of 'Jambu' (*Syygium Jambolaum*) before the starting of the urine collection

The potency of the tablets reported to contain 50 mg of ascorbic acid, was checked by tests in Lahore and the Nutrition Research Laboratories Coonoor Values obtained for tablets ranged from 45 mg to 50 mg

SUMMARY

An experiment was carried out to discover the effect of ascorbic acid on gingivitis and associated conditions in Indian children in an orphanage One hundred mg of ascorbic acid

was given to 50 children for 100 days a similar control group receiving no supplement .No change in gingival conditions was observed as the result of giving ascorbic acid

ACKNOWLEDGMENT

Grateful thanks are due to Dr T J Thompson Wells Roche Scientific Division, Volkart Brothers Bombay for the free supply of the ascorbic acid used in these experiments

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Ind Jour Med Res 31, 2 October 1943

THE EFFECT OF 'EXERCISE' ON THE PYRUVIC ACID CONTENT OF NORMAL AND VITAMIN-B_1 DEFICIENT RICE-MOTH LARVÆ (*CORCYRA CEPHALONICA* ST.)

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[Received for publication September 2, 1943.]

It has been established beyond doubt that accumulation of pyruvic acid takes place in the blood of man and certain experimental animals as a result of thiamin deficiency. The relation between pyruvic acid accumulation in the blood, the amount of exercise and vitamin-B_1 intake has been investigated by several workers. Thus Johnson and Edwards (1937) found that in normal subjects, who were exercised to exhaustion, pyruvic acid appeared in the blood in amounts similar to those which are reached in severe beri-beri. Lu and Platt (1939) reported that light muscular work by vitamin-B_1 deficient human subjects was followed by an increase in blood pyruvate. During rest after exercise values returned to the initial level in normal subjects in less than an hour, whereas in vitamin-B_1 deficient subjects return was delayed. Lu (1939) also studied the effect of muscular activity on the blood pyruvate of rabbits and found that the value rose as a result of exercise but returned to the original value in about half an hour. Bollman and Flock (1939) investigated the effect of exercise on pyruvic acid in the blood and muscles of normal and thiamin-deficient rats. They found that though the blood and muscles of thiamin deficient rats contained more pyruvate than those of normal rats, the increase of pyruvate in contracting muscle was of the same order in both normal and deficient rats. Table I summarizes the results obtained by different workers in the field —

TABLE I

The effect of 'exercise' on pyruvic acid in the blood and muscle of normal and thiamin-deficient human beings, rabbits and rats

(Pyruvic acid in mg. per 100 g. or ml.)

Subject	Initial level	Immediately after exercise	3 minutes after	16 minutes after	½ hour after	40 minutes after	Author
Man (blood) —							
Normal	0·18			0·60	0·40	0·27	Johnson and Edwards (1937)
Normal (cured cases)	0·53	0·79			0·56		Lu and Platt (1939)
Sub acute cases	0·76	0·85			0·87		Lu and Platt (1939)
Rabbit (blood)	1·34		4·68		1·34		Lu (1939)
Rat (muscle) —							
Normal	1·52	4–6	A gradual return in 2 to 5 minutes				Bollman and Flock (1939)
B_1 deficient	3·30	4–6					Bollman and Flock (1939)

Sarma and Bhagvat (1942) showed that an accumulation of pyruvic acid occurs in rice-moth larvæ (*Corcyra cephalonica* St.) which have been reared on a thiamin-deficient diet. This disappears on the inclusion of vitamin B_1 in the diet. The relation between thiamin intake and the accumulation of pyruvate in the larvæ was closely analogous to that observed in human beings, rats, rabbits and pigeons. It was felt to be of interest, therefore, to study the changes taking place in the pyruvic acid content of larvæ, both normal and B_1 deficient, when they were subjected to strenuous 'exercise'.

Experimental —The technique of handling and rearing of the rice-moth larvæ has been described by Bhagvat and Sarma (1943) in a previous paper The larvæ which were reared for ten days on whole wheat were transferred to a thiamin-deficient diet, which had the following composition —

	Grammes
Purified starch	60
Autoclaved yeast	10
Purified casein	18
Salt mixture (McCollum and Davis)	4
Sugar	4
Shark-liver oil	2
Coco-nut oil	2
Total	100

When the larvæ developed thiamin deficiency, indicated by the accumulation of pyruvic acid in a representative batch of larvæ, the remaining larvæ were used to investigate the effect of exercise on their pyruvic acid content Normal healthy larvæ were taken from the whole-wheat diet and were simultaneously investigated The larvæ in each case were divided into several batches of 7 to 10 larvæ, and their weights recorded The larvæ were 'exercised' by touching the hind part of the body by a soft paint brush Each touch or push made the larva move forwards in a jerk Fifty pushes were given as rapidly as possible Larvæ were then taken at specified intervals and crushed in 10 per cent trichloracetic acid Their pyruvic acid content was determined by the method already described (Sarma and Bhagvat, *loc cit*) Table II gives the results obtained with normal larvæ, thiamin-deficient larvæ, and larvæ which after the development of thiamin deficiency were fed on the basal diet plus 5 μg of thiamin per gramme of diet for a period of 10 days —

TABLE II

Effect of 'exercise' on the pyruvic acid content of normal and thiamin-deficient larvæ

(Mg of pyruvic acid per 100 g of dry weight)

Initial level	3 minutes after exercise	30 minutes after exercise	45 minutes after exercise	1½ hours after exercise	3 hours after exercise	5 hours after exercise
Normal larvæ						
29 80	35 90	31 40	30 80	30 10	30 00	
27 42	35 50	33 10	32 88	27 60	27 36	
Thiamin-deficient larvæ						
78 32	100 44	92 16		85 16	82 04	79 60
82 64	99 24	90 68		83 96	82 00	
165 52	177 08	168 80		164 00	161 60	
Thiamin-deficient larvæ subsequently fed on the basal diet + 5 μg g of B_1 for ten days						
33 45	38 70	36 90		35 45	34 10	
36 80	41 30	40 50		38 70	37 08	

Table II shows that there was an increase in the pyruvic acid content of both normal and thiamin deficient larvæ after exercise Similar results are obtained with larvæ which had been cured of thiamin deficiency The highest pyruvic acid values were found in larvæ tested for three minutes after exercise The time taken for pyruvic acid values to return to the original level was substantially the same in normal and thiamin-deficient larvæ, i e the curve of pyruvic acid response to 'exercise' was not influenced by thiamin deficiency These results are in good agreement with those of Bollman and Flock (*loc cit*), who observed no great difference in the pyruvic acid changes in the exercised muscles of thiamin deficient and normal rats The pyruvate changes in the 'exercised larvæ do, however, differ from those obtained in the case of blood by different workers, in thiamin deficiency blood-pyruvate values take a time longer than the normal to return to the initial level The results obtained with the larvæ cannot, however, strictly be compared with those obtained with blood It is believed that pyruvic acid is formed in muscle during exercise and diffuses thereafter into the blood stream Larval tissue is more closely analogous to muscle than to blood

It is interesting to note the close similarity in the biochemical functions of thiamin in such widely different organisms as the rice moth larva and *homo sapiens* It indicates how closely thiamin is bound up with carbohydrate metabolism and suggests that its functions are the same in all organisms to which it is essential

Summary

1 An increase was observed in the pyruvic acid content of normal and thiamin-deficient larvæ, subjected to a period of strenuous 'exercise' The highest pyruvic acid values were found in larvæ tested three minutes after 'exercise'

2 The time taken for pyruvic acid values to return to the original level was three hours and was substantially the same in normal and thiamin-deficient larvæ

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Ind Jour Med Res **31**, 2 October 1943

RIBOFLAVIN AND PYRIDOXIN (VITAMIN B_6) AS GROWTH-PROMOTING FACTORS FOR RICE-MOTH LARVÆ (*CORCYRA CEPHALONICA* ST.)

BY

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[Received for publication, September 2, 1943.]

STUDY of the nutritional requirements of insects has revealed that like man and other mammals, they need various components of the vitamin B complex for normal metabolism and growth. A considerable amount of work has been done on the growth-promoting factors needed by the flour beetle *Tribolium confusum* (Sweetman and Palmer, 1928; Barton-Wright, 1941; Frankel and Blewett 1942; Rosenthal and Reichstein, 1942). It has been found that the tribolium requires thiamin, nicotinic acid amide and biotin for normal growth. Van t Hoog (1936) showed that thiamin and riboflavin are essential for the growth of the fruit fly (*Drosophila melanogaster*). The chemical nature of the growth factors required by the yellow-fever mosquito (*Ædes ægypti*) was investigated by Subbarow and Trager (1939). They found that for the normal development of the mosquito, flavine or flavine-purine complex, pyridoxin, pantothenic acid and glutathione were necessary. Rubenstein and Sekhun (1939) showed that the growth of the larvæ of galleria on a diet low in nicotinic acid was influenced by the amount of nicotinic acid added to the diet.

The vitamin requirements of the rice-moth larvæ (*Corcyra cephalonica* St.) have been studied within recent years. Swamy and Sreenivasaya (1939) first suggested the use of rice-moth larvæ as test animals for vitamin studies. Sarma, Swamy and Sreenivasaya (1942) showed that the larvæ require vitamin B_1 for growth and that under certain conditions growth was proportional to the amount of vitamin B_1 present in the diet. It was also demonstrated that the larvæ require a fat-soluble factor which is of the nature of a sterol (Sarma and Sreenivasaya, 1941). In the present communication it is shown that the rice-moth larva requires riboflavin and pyridoxin (vitamin B_6) for growth.

EXPERIMENTAL

The choice of a basal diet.—Experiments were first carried out with the object of finding a suitable basal diet, which would not support the growth of the larvæ except on the addition of pure water-soluble vitamins. It had been found earlier that the larvæ grew well on whole wheat and it appeared feasible to prepare a satisfactory basal diet from whole wheat by depriving it of the water-soluble vitamins without otherwise materially altering its chemical composition. This, it was found by trial could not be achieved either by extraction of whole wheat with acid or its digestion with pepsin. The method which was finally adopted consisted of the extraction of whole-wheat powder with 3 per cent. sodium chloride solution together with autolysis. Riboflavin is easily removed by the process, as also vitamin B_6 which according to Birch and Gyorgy (1936) is removed to the extent of 80 to 100 per cent. by autolysis alone.

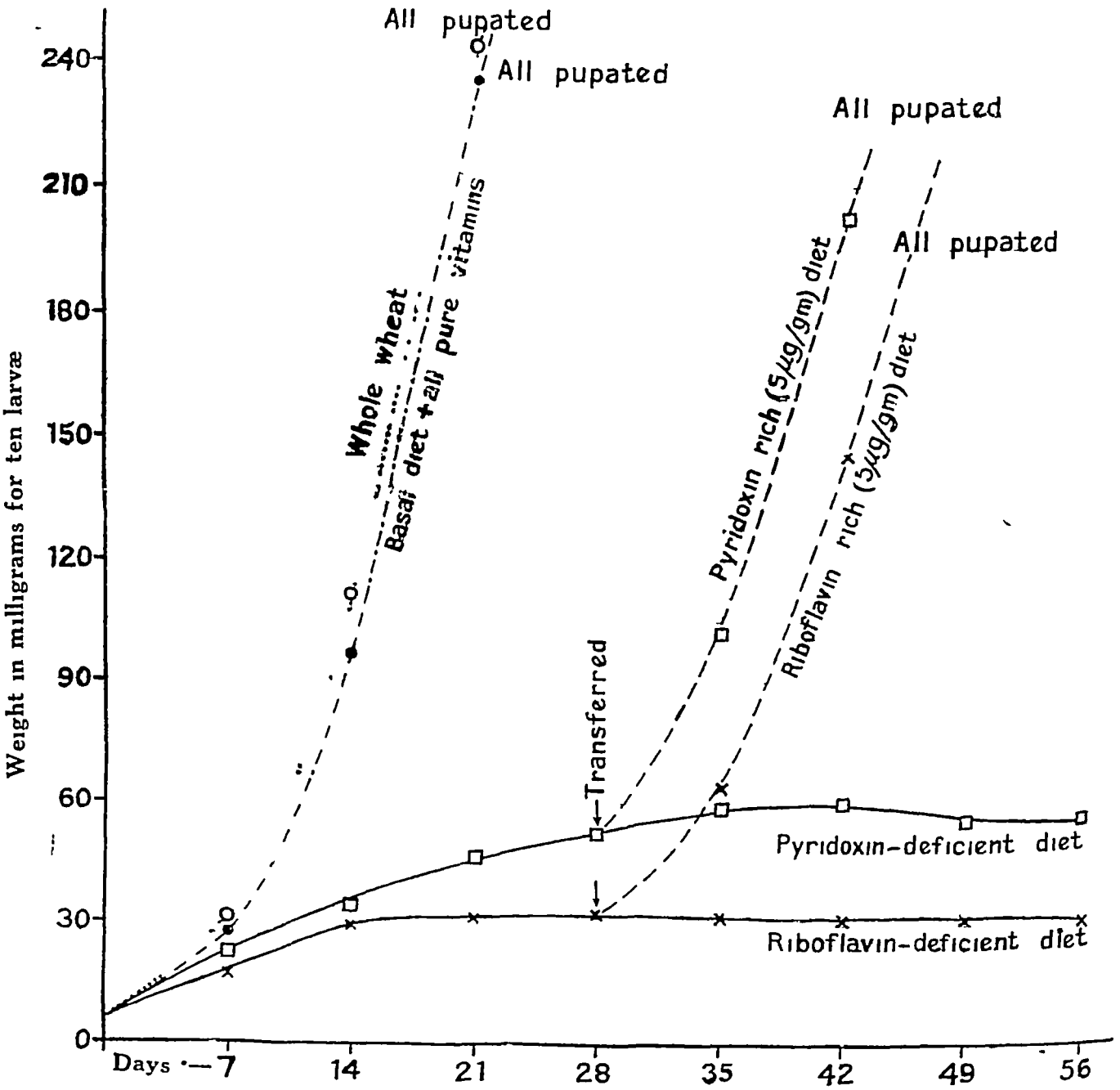
Preparation of the basal diet.—One hundred grammes of whole wheat powder was stirred with one litre of 3 per cent. sodium chloride solution. One ml. each of chloroform and toluene respectively were added to the mixture, and the whole solution was kept at room temperature for a period of 24 hours with occasional stirring. The supernatant, yellow in colour was discarded and the process repeated three times, after which the residue was washed with a litre of water to remove the salt. After standing overnight the supernatant was discarded and the wheat residue was filtered on a Buchner funnel and dried.

TABLE II.

Weight in milligrams for ten larvæ

	Diet	28 days	35 days	42 days	49 days	56 days
A	Basal diet	11 8	11 7	11 5	11 6	11 3
A_1	Basal diet + thiamin + pyridoxin + nicotinic acid + calcium pantothenate + riboflavin	11 8	20 0	93 9	196 8	All pupated
B	Riboflavin deficient diet	24 1	23 5	23 8	24 3	24 0
B_1	Diet same as A_1	24 1	50 4	130 1	211 4	All pupated
C	Pyridoxin deficient diet	41 6	45 4	43 4	42 6	42 5
C_1	Diet same as A_1	41 6	84 7	165 1	228 4	All pupated

GRAPH 2



Graph 2.—The effect of pyridoxin and riboflavin on the growth of larvæ

These experiments were reported several times with similar results Graph 2 represents the cessation of growth of the larvæ when on riboflavin and pyridoxin-deficient diets and its resumption as soon as the larvæ are transferred to diets containing those vitamins The growth curves after the addition of these vitamins resemble those of larvæ on the whole wheat diet

In a further series of experiments diets were prepared in which riboflavin in solution was destroyed by irradiation and by heating with alkali Five μg of riboflavin solution were exposed to ultra violet light for 30 minutes and the solution subsequently mixed with 5 g of the riboflavin deficient diet Another solution containing 5 μg of riboflavin was boiled with 0 5 c c of 2 N alkali for 5 minutes It was then cooled and neutralized with alkali The resultant solution was then mixed with 5 g of the riboflavin deficient diet

Larvæ which were kept on a riboflavin deficient diet for a period of 28 days were taken divided into several batches and placed on the diets shown in Table III Their weights are also given in Table III —

TABLE III

Weight in milligrams for ten larvæ

	Diet	Initial weight	7 days	14 days	21 days	28 days
A	Riboflavin deficient diet	24 1	25 3	25 3	26 1	25 5
B	Riboflavin deficient diet + 5 μg of riboflavin solution exposed to ultra violet light for 30 minutes	25 3	25 8	26 0	26 0	25 7
C	Riboflavin deficient diet + 5 μg of riboflavin solution treated with alkali and neutralized	25 7	26 1	26 3	26 3	26 1
D	Riboflavin deficient diet + 1 μg of riboflavin per g of diet	25 6	46 2	89 7	128 0	All pupated

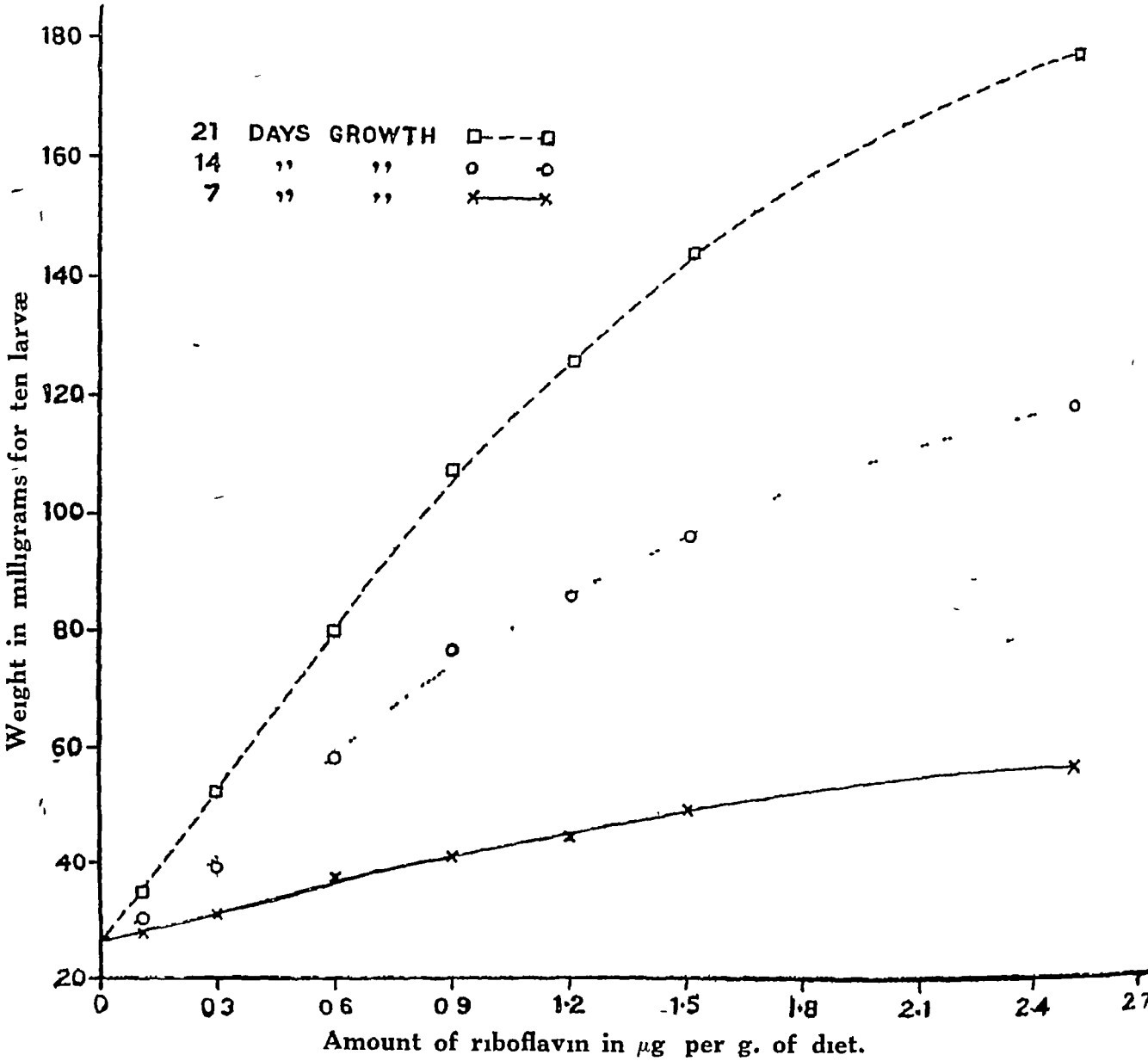
The results show that growth took place only on the inclusion of riboflavin in the diet and that decomposed products of riboflavin which are present in the solution after irradiation or destruction with alkali have no growth-promoting effect

For experiments on the effect of riboflavin on growth, larvæ which have been reared on the whole-wheat diet for a period not exceeding 8 to 10 days should be used If bigger larvæ are taken from stock they do not stop growing when put on the riboflavin-deficient diet and many may reach the stage of pupation

Effect of different amounts of riboflavin in the diet on the growth increase of riboflavin deficient larvæ —It was of interest to investigate the relation between the growth response of the riboflavin-deficient larvæ and the amount of riboflavin in the diet Several diets were prepared containing quantities of pure riboflavin ranging from 0 1 μg to 5 μg per g of diet Larvæ reared on a riboflavin deficient diet for a period of 28 to 30 days were put on these diets and their weights recorded after 7 days, 14 days and 21 days respectively Graph 3 shows graphically the relation between larval growth and the concentration of riboflavin in the diets

It will be seen from the graph that a linear relationship was observed between larval growth and the concentration of riboflavin in the diet up to about 1 2 μg of riboflavin per g of diet, but thereafter there was a flattening of the curves The growth of the riboflavin-deficient larvæ on the diet containing 5 μg of riboflavin per g of diet was more or less the same as that on diets containing 2 5 μg per g of diet The maximum growth of the larvæ was reached on diets containing 2 5 μg of riboflavin per g of diet and higher concentrations did not appreciably increase the growth rate

GRAPH 3.



Graph 3 —Growth response to varying amounts of riboflavin

Preliminary experiments were carried out to discover whether the riboflavin-deficient larvæ could be used to estimate riboflavin present in foodstuffs and biological materials It was found by trial that the optimum range of concentration of riboflavin to work with varied between 0 μg and 1 2 μg of riboflavin per g of diet, since the growth response of the deficient larvæ to different amounts of riboflavin within these limits was most marked Riboflavin was extracted by the usual methods and incorporated in the riboflavin-deficient diet in such amounts that the concentration of riboflavin in the diet was within the range given above

Five grammes of Torula yeast (*Torula utilis*) were extracted by the method of Hodson and Norris (1939) and an aliquot of the extract was mixed with the riboflavin-deficient diet Cow's milk was mixed directly with the deficient diet in suitable amounts Test materials were also prepared by adding pure riboflavin to yeast extract and cow's milk to discover whether any destruction of riboflavin takes place during the experiments No appreciable loss took place Riboflavin-deficient larvæ were put on the several diets and their weights recorded after 7 and 14 days respectively From the curves given in Graph 3, the amounts of riboflavin

present in Torula yeast and cow's milk were found to be as follows Torula yeast, 33 2 μg per g and cow's milk 1 9 μg per c c These results agree fairly well with those obtained by other workers using other methods

The larval method of assay can usefully be employed to estimate riboflavin in various substances It is highly unlikely that colouring matter in the riboflavin extracts, which seriously interferes with the fluorimetric estimation of riboflavin, will influence the growth of riboflavin deficient larvæ The larval method is probably simpler than the micro-biological method of Snell and Strong (1939) in which bacterial contamination must be strictly avoided and inhibiting substances removed to get accurate results

Discussion

The larvæ which are riboflavin and pyridoxin deficient may serve as excellent material for a study of the biochemical and physiological changes which occur in the living organism as a result of such deficiencies The deficient larva could also be used for histological studies A study of the body composition of the larvæ fed on diets containing varying amounts of riboflavin and pyridoxin might give interesting results

The results further indicate that the rice moth larva does not apparently need the other members of the vitamin-B_2 complex for growth But this must be confirmed by devising a basal diet which is absolutely free from those vitamins Biotin for instance is one of the components of the vitamin-B_2 complex which is not easily removed by the ordinary methods of extraction, and it is not clear from the experiments recorded in this paper whether the larvæ need biotin for their growth

Summary

1 The rice moth larva (*Corcyra cephalonica* St) requires riboflavin and pyridoxin (vitamin B_6) for its growth It apparently does not need nicotinic acid and pantothenic acid

2 The growth of riboflavin-deficient larvæ was proportional to the amount of riboflavin added to the diet up to 1 2 μg per g of diet Larger amounts of riboflavin did not accelerate growth

3 Rice-moth larvæ can be used for the estimation of riboflavin in foodstuffs and biological materials

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Ind Jour Med Res, 31, 2, October 1943

ACCUMULATION OF PYRUVIC ACID IN RICE-MOTH LARVÆ (*CORCYRA CEPHALONICA* ST) FED ON A VITAMIN B_1-DEFICIENT DIET

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[Received for publication, April 8, 1943]

It is now fully established that when experimental animals such as the rat or the pigeon are kept on a vitamin B_1-deficient diet there occurs in the blood a marked accumulation of carbonyl compounds chiefly pyruvic acid (Thompson and Johnson 1935, Li and Kato, 1940) This characteristic change has also been observed in the blood urine and cerebrospinal fluid of persons suffering from beri beri (Platt and Lu 1936) In all these cases administration of a suitable amount of vitamin B_1 brings about a rapid fall in the pyruvic acid content of the blood to the normal level A biochemical process of the same nature has been observed in lower forms of life, e g in the fungus of *P blakesleeanus* Haag (1940) has shown that in the absence of vitamin B_1 there was an accumulation of pyruvic acid in a glucose medium inoculated with spores of *P blakesleeanus* Haag and Dalphin (1940) also demonstrated the production of pyruvic acid when a medium devoid of vitamin B_1 was inoculated with yeast and kept at 25°C for 24 hours

In view of these observations it was felt to be of interest to discover whether the larva of common rice moth (*Corcyra cephalonica* St) for whose normal growth vitamin B_1 has been found indispensable (Swamy and Sreenivasaya 1940) shows similar biochemical reactions An additional object in taking up this investigation was to see whether the larvæ could be employed for the biological determination of vitamin B_1 The use of insect larvæ for such a purpose would have the advantage over the use of large experimental animals such as the rat and the pigeon, in that it would effect a considerable economy of time (the life-span of the larvæ being short) and also of experimental material (purified diets, synthetic vitamins etc) Further the ease with which the larvæ can be grown and handled and the rapid reproducibility of results are other points in favour of such a choice

The rice moth is a common species usually found in godowns where rice and other grains are stored It belongs to the class of Lepidopterous insects and was first studied in detail by Krishna Ayyar (1934) It is a very destructive pest in grain stores in South India, damaging materials like cholam (*Sorghum vulgare*) wheat, wheat bran, rice rice bran, Bengal gram, ground-nut seed, etc In general under conditions favourable to its development no material in storage escapes its attention

The life cycle of the rice moth from egg-larvæ-pupæ-moth lasts about 40 to 60 days, the period varying with the temperature, humidity and the grain on which larvæ feed It feeds only during its larval period during which it undergoes eight moulting changes If the larvæ are transferred from one cereal to another, e g from millet to wheat or wheat to rice during the period of growth they will continue to feed equally readily

The vitamin requirements of various species of insects have been studied within recent years A few suggestions have been made that insects might be used for vitamin assays Thus, Rubenstein and Sekhun (1939) have suggested the use of the larvæ of galleria for a sensitive biological test for detecting minute amounts of nicotinic acid Swamy and

Sreenivasaya (1939) have advocated the use of rice-moth larvæ as test animals for vitamin studies In a later publication, Sarma Swamy and Sreenivasaya (1942) have shown that the growth of the larvæ was proportional to the amount of vitamin B_1 present in the diet

Experimental

Preparation of the diet —The basal diet used throughout the experiments had the following composition —

	Grammes
Purified starch	60
Autoclaved yeast	10
Purified casein	18
Salt mixture (McCollum and Davis)	4
Sugar	4
Shark-liver oil	2
Coco-nut oil	2
Total	100

The casein and starch used in the preparation of the basal diet must be carefully purified Even traces of vitamin B_1 in the diet will prevent the development of vitamin B_1-deficiency If such traces are present the accumulation of pyruvic acid in the larvæ will be small, even when the larvæ are fed on a deficient diet for a long time

Starch was purified by keeping it for 48 hours in contact with 10 volumes of 0 2 per cent alkali It was washed first with acidulated water, then with water and finally with alcohol It was then dried either in the sun or in a vacuum desiccator Casein was purified by repeated dissolution in alkali at 100°C and precipitation with dilute acid The precipitated casein was washed free from acid and then with alcohol and dried in the sun

Autoclaved yeast was prepared by autoclaving dried Torula yeast (grown on molasses) at 15-lb pressure for 6 hours in layers not exceeding $\frac{1}{4}$ inch and subsequent drying in the sun

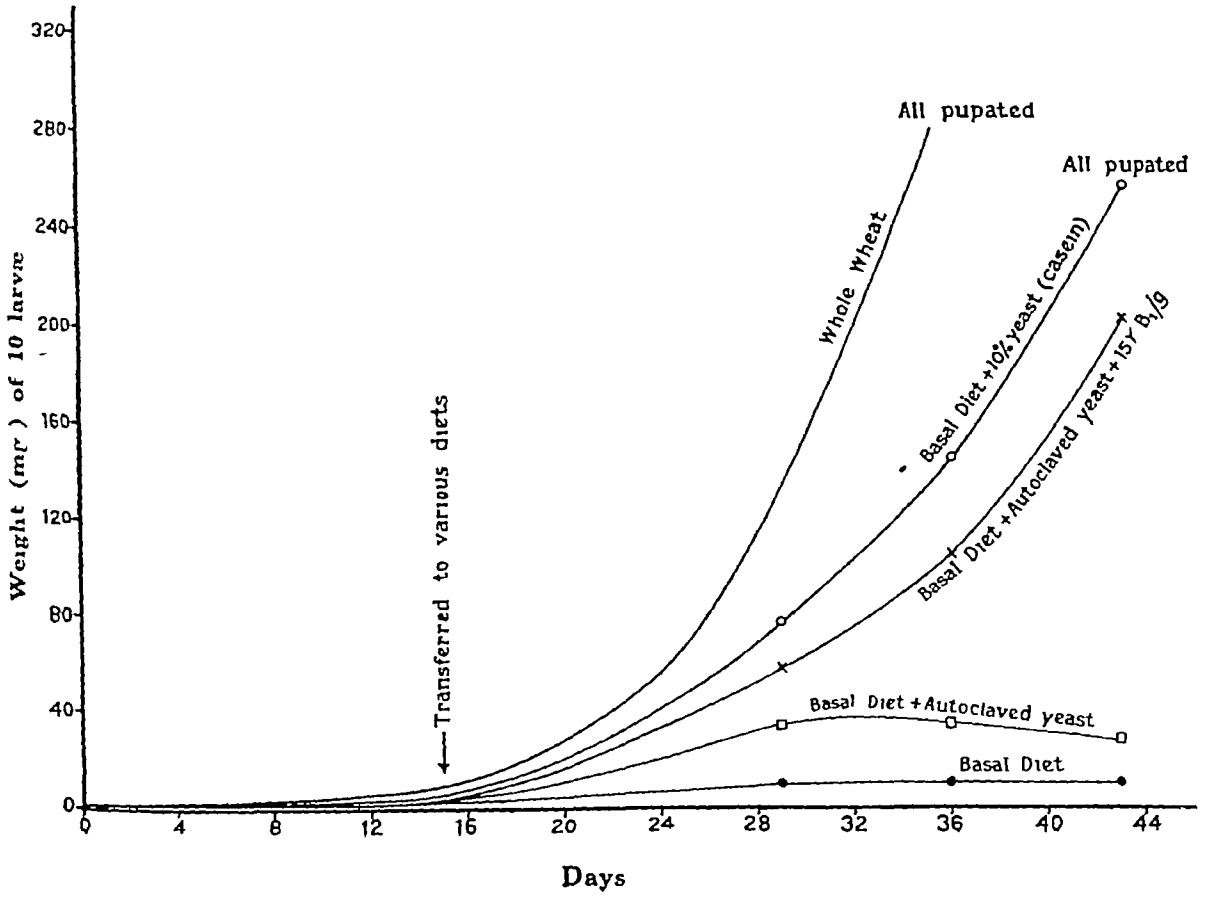
Proteins from Bengal gram (*Cicer arietinum*) and green gram (*Phaseolus radiatus*) were prepared by extracting the powdered pulse with 5 per cent saline and dialysing the extract Albumin in the supernatant fluid was precipitated with dilute acetic acid and was added to the total globulins The proteins were washed with water, then with alcohol and ether and dried in a vacuum desiccator over sulphuric acid Total wheat proteins were prepared by extracting whole-wheat meal with 0 2 per cent alkali and precipitating the proteins with acetic acid They were washed free from the acid and dried in a desiccator

The different constituents of the basal diet were ground up with a little water and then granulated through a 30-mesh to the inch sieve The granulated material was dried in a desiccator over sulphuric acid and preserved in bottles

Rearing and feeding technique —As soon as the eggs hatched out the larvæ were carefully picked up by a camel-hair brush and put on a diet of whole-wheat flour If they are given a vitamin B_1-deficient diet immediately after hatching, the mortality is very high Sarma and Sreenivasava (unpublished results) have observed that they grow better on whole wheat than on any other cereal The larvæ were kept in a large Petri-dish (6″ diameter and $\frac{3}{4}$″ height) in the incubator at 30°C with a humidity of 75 per cent After 15 days, they were removed from the flour by means of a thin glass-rod, care being taken to see that none was injured A careful selection of the larvæ was then made so that only those of a fairly uniform size were used for experiment They were gently cleaned with a camel-hair brush in order to remove

any adhering traces of wheat flour After a representative sample of 20 larvæ had been taken for weighing they were transferred to a small-sized Petri dish (1" diameter and $\frac{3}{4}$" height), and the experimental diets were sprinkled on them in excess of their requirements Ten larvæ were taken at different intervals of time and weighed on a microbalance The increase in growth of the larvæ when fed on different diets is shown graphically in Fig 1 —

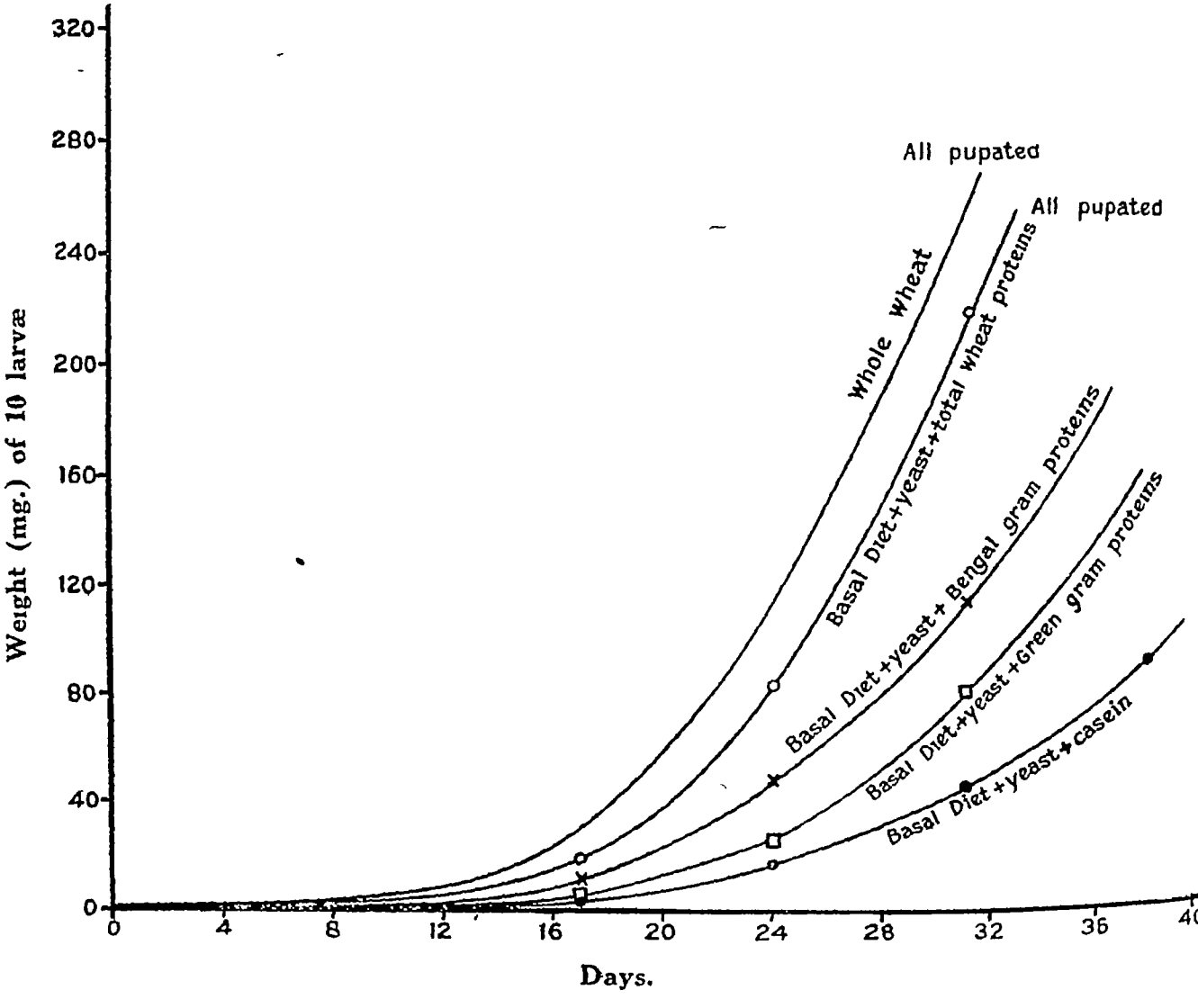
Fig 1



It will be seen from Fig 1 that the larvæ did not grow when fed on the basal diet alone or the basal diet + autoclaved yeast When, however, the diet was supplemented with vitamin B_1 or unautoclaved yeast the growth response was good (*cf* Sarma, Swamy and Sreenivasaya *loc cit*), though it did not equal that of the larvæ fed on whole-wheat flour Further experiments suggested that the better growth on the whole-wheat diet was due, not to the presence in wheat of some unidentified growth factor or factors, but to the superiority of wheat protein in supporting larval development Casein was replaced by various proteins (at 5 per cent level) in the basal diet The growth of the larvæ when fed on the basal diet containing unautoclaved yeast and total wheat proteins compares well with that of larvæ on whole

wheat (Fig 2) Of the proteins studied casein seemed to be the least effective in promoting growth In all subsequent experiments, however, casein was used because of the ease with which it can be prepared and purified The main object of the experiments was to study the metabolism of pyruvic acid which is not dependent on the protein used in the basal diet

FIG. 2.



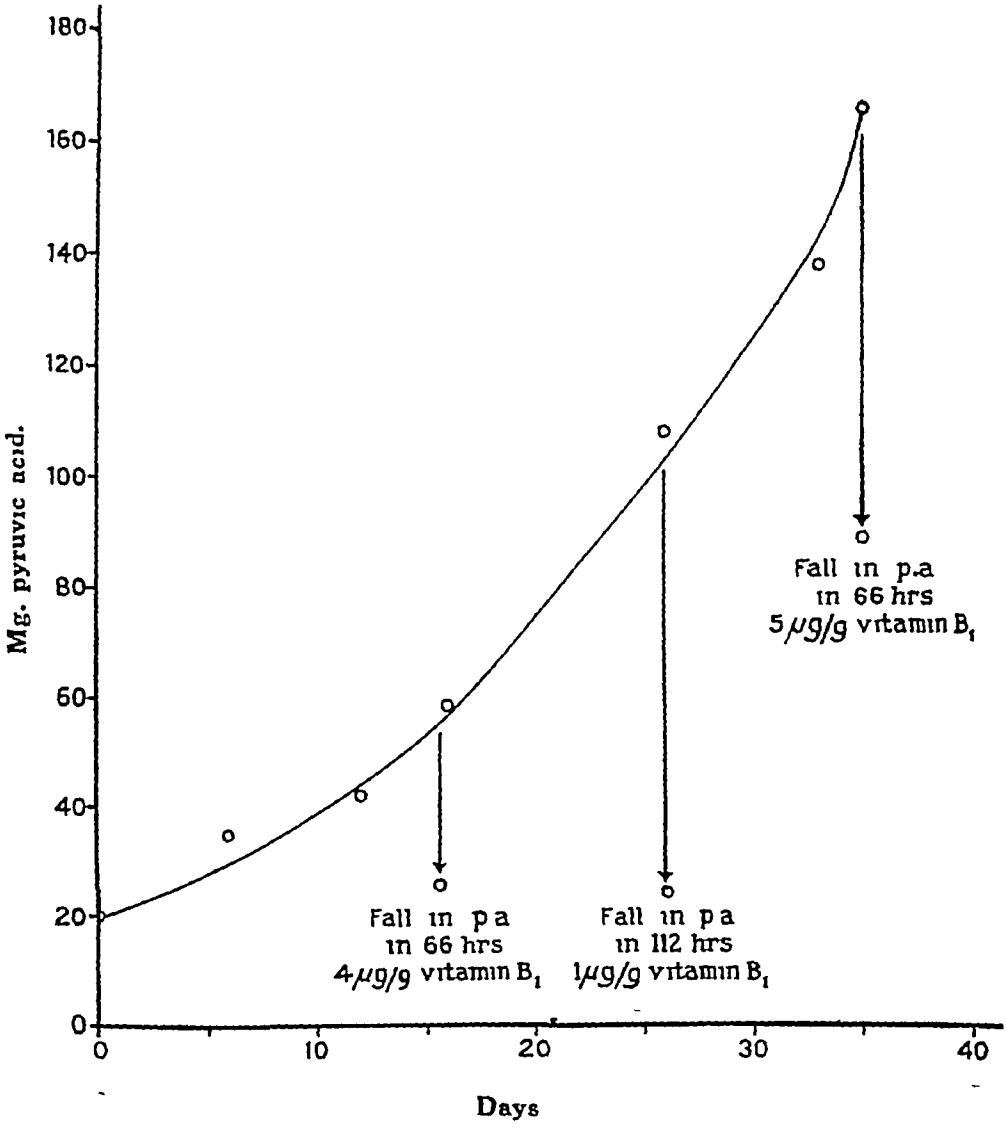
Accumulation of pyruvic acid in larvæ fed on a vitamin B_1-deficient diet—The technique followed in these experiments was as previously described The larvæ were first fed on a whole-wheat diet for 15 days, after which they were transferred to a diet deficient in vitamin B_1 At this stage 10 larvæ weighed from 8 mg to 22 mg A representative sample of 10 larvæ was weighed at intervals When the larvæ were placed on the vitamin B_1-deficient diet they continued to show slight increase in weight for about 15 days, after which their weight became stationary

Pyruvic acid was estimated at various intervals of time by the method of Lu (1939), a photo-electric colorimeter constructed in the laboratories being employed From 8 to 15 larvæ weighing 20 mg to 75 mg as a group were taken for the estimation of pyruvic acid The

effect of transferring larvæ fed for various intervals of time on the vitamin B_1-deficient diet to diets containing 1 μg., 4 μg. and 5 μg. of vitamin B_1 per g. was investigated. Fig. 3 shows the increase in the pyruvic acid content of larvæ based on tests carried out on representative samples of 10 larvæ at various stages of deficiency. The larvæ used for the estimation of pyruvic acid after administration of vitamin B_1 were samples of the batches of larvæ on which

Fig. 3

Accumulation of pyruvic acid.



the ascending curve (Fig. 3) is based. Table I shows the pyruvic acid content of larvæ on whole wheat and on the basal diet with and without vitamin B_1. For purposes of comparison it also includes some blood pyruvic acid values for the blood of normal and vitamin B_1-deficient rats, pigeons and human beings as reported by other workers.

TABLE I.

Pyruvic acid in the tissues of normal and vitamin B_1-deficient larvæ and in the blood of normal and vitamin B_1-deficient rats pigeons and human beings

		Pyruvic acid	Author
1	Larvæ on whole wheat	185 to 205 mg /100 g of dry weight	Sarma and Bhagvat (1942)
2	Larvæ on vitamin B_1 deficient diet for 35 days	164 3 mg /100 g of dry weight	
3	Larvæ from (2) transferred to a diet containing 5 μg vitamin B_1 per g for 66 hours	88 25 mg /100 g of dry weight	
4	Pigeon's blood, normal	0 84 mg /100 c c	Thompson and Johnson (1935)
	,, ,, deficient	5 85 ,,	
5	Rat blood, normal	0 96 ,,	Li and Kato (1940)
	,, ,, deficient	5 62 ,,	
6	Human blood, normal	0 56 ,,	Lu (1939)
	,, ,, deficient	2 35 ,,	

It will be seen that the larvæ, like man and the laboratory animals mentioned, accumulate pyruvic acid when fed on a vitamin B_1-deficient diet The amount of pyruvic acid present was reduced on the inclusion of vitamin B_1 in the diet and the larvæ showed an increase in weight In these particular experiments, however, the percentage disappearance of pyruvic acid was not proportional to the amount of vitamin B_1 added to the diet One of the reasons for this was that the vitamin B_1 was not thoroughly mixed into the diet Subsequently it was found that when the vitamin was thoroughly mixed with the diets by grinding the latter with the requisite amount of a solution of vitamin B_1 plus a little water prior to granulation and dehydration a greater fall in pyruvic acid could be obtained with a smaller concentration of vitamin B_1

It is to be observed that the amount of pyruvic acid present in the larvæ, both in the normal and deficient states, was much larger than that present in the blood of man, rat or pigeon

It was found that the accumulation of pyruvic acid was influenced by the stage at which the larvæ were taken from the whole-wheat diet and placed in the experimental diet When larvæ weighing more than 25 mg per 10 were taken, the accumulation of pyruvic acid in a given period was much less than in the case of larvæ of lower initial weight This point is illustrated in Table II The explanation is presumably that the larger larvæ were able to lay in a store of vitamin B_1 during their longer period on the whole-wheat diet The best results were obtained when the initial weight of larvæ was 8 mg to 20 mg per 10 and larvæ of this size were used in subsequent experiments

TABLE II

Effect of initial weight of the larvæ on the accumulation of pyruvic acid

Initial weight of 10 larvæ, mg	Fed on vitamin B_1 deficient diet for in days	Mg pyruvic acid per 100 g of dry weight
27 9	22	55 10
30 2	15	24 85
5 2	26	108 00
17 3	16	57 94

Larvæ which were grown on whole wheat flour for 16 days were transferred to different diets in groups of 15. They were weighed weekly and their pyruvic acid content was determined after 21 days. The results are given in Table III —

TABLE III

Effect of minute amounts of vitamin B_1 on the growth and pyruvic acid content of larva.

Diet	Weight of 15 larvæ (mg.)				Mg. pyruvic acid/100 g. of dry weight
	Days				
	0	7	14	21	
1 B. D. + A. Y. — 0.05 μg. vitamin B_1 per g.	33.0	75.75	163.0	172.65	18.0
2 B. D. + A. Y. — 0.1 μg. vitamin B_1 per g.	32.7	63.20	157.75	205.20	20.4
3 B. D. + A. Y. — 0.5 μg. vitamin B_1 per g.	31.8	72.51	142.8	213.75	20.43
4 B. D. + A. Y. — 1.0 μg. vitamin B_1 per g.	37.1	77.18	183.3	277.0	20.8
5 B. D. — A. Y. + 5.0 μg. vitamin B_1 per g.	32.3	78.94	195.05	261.0	14.0
6 B. D. + A. Y.	17.3			60.33	79.5
7 B. D. + A. Y. + 0.2 c.c. rat's blood	26.9	61.1	133.0	162.3	32.64
8 Whole wheat	30.0	133.0			19.26

B. D. = Basal diet. A. Y. = Autoclaved yeast.

Table III shows that even minute amounts of vitamin B_1 were sufficient for the maintenance of proper growth and for the prevention of the accumulation of pyruvic acid in the larvæ. An additional confirmation of this observation was found when purified and unpurified tapioca starch was used in the preparation of the basal diet. The unpurified starch presumably contained only a trace of vitamin B_1 and yet that amount was sufficient to prevent the accumulation of pyruvic acid (Table IV). In experiments of this nature it is necessary to use purified starch.

TABLE IV

The effect of using purified and unpurified starch on the accumulation of pyruvic acid

	Mg. pyruvic acid/100 c. of dry weight	
	Found	Theoretical figure as calculated from the graph in Fig. 3
1 Larvæ on purified starch	(1) 112.0 (2) 115.0	120.0
2 „ unpurified starch	(1) 85.63 (2) 80.63	120.0 155.0

Fair growth took place on the diet containing rat's blood (Table III) and accumulation of pyruvic acid was low, indicating that the amount of blood included supplied sufficient, or almost sufficient, vitamin B_1 to cover the needs of the larvæ In a further experiment 0 2 c c of pigeon's blood was added to the diet This was obtained (*a*) from a normal pigeon, (*b*) from a pigeon suffering from 'polyneuritis' induced by feeding a diet of raw washed machine-milled rice for 4 weeks, and (*c*) from the same pigeon after the 'polyneuritic' signs had disappeared following the intravenous injection of vitamin B_1 In another experiment human milk was added to the diet These diets were fed for 70 hours to larvæ in a state of vitamin B_1 deficiency The blood from the polyneuritic pigeon induced only a slight reduction in pyruvic acid, while the other supplements caused or induced a greater reduction The results of these experiments are shown in Table V —

TABLE V

Fall in pyruvic acid on transferring deficient larvæ to diets containing blood and milk

		Mg pyruvic acid/100 c of dry weight	
		Initial	After 70 hours
1	Basal diet	121 92	140 56
2	Basal diet + 0 2 c c normal pigeon's blood	121 94	76 00
3	Basal diet + 0 2 c c polyneuritic pigeon's blood	121 94	108 40
4	Basal diet + 0 2 c c blood from polyneuritic pigeon after relief of symptoms on vitamin B_1 injection	121 94	66 20
5	Basal diet + 0 2 c c human milk (normal)	173 12	87 80

These preliminary experiments suggest the possibility that the larvæ might be used for the detection of minute amounts of vitamin B_1 present in blood and other biological materials and incidentally for the study of avitaminosis B_1 in man

Discussion

These experiments indicate that the biochemical rôle of vitamin B_1 in the insect species studied is essentially the same as in man and in other mammals The parallelism in this respect between such widely different organisms is of great biological interest It suggests that vitamin B_1 is essential to all forms of life, except perhaps the most lowly, and that the biochemical processes with which it is concerned are of fundamental importance in the metabolism of living tissue The technique described is susceptible of development in several directions for the study of the functions of the vitamin The larvæ are small and easy to handle, and experimental results can be easily and rapidly reproduced Mention was made above of the possibility of using the larvæ to detect minute amounts of vitamin B_1 Another possibility is their use in investigating changes in the milk of mothers whose infants develop acute beri-beri Some workers hold the view that infantile beri-beri is essentially an intoxication, due to the presence in the maternal milk of a toxic factor which is the product of deranged carbohydrate metabolism in the mother The feeding of such milk to larvæ might throw light on the problem

SUMMARY.

1 A technique for handling and rearing rice moth larvæ is described The larvæ will grow normally on a basal diet consisting of wheat protein yeast salt and shark-liver oil

2 When the larvæ are fed on a vitamin B_1 deficient diet, they accumulate pyruvic acid like man and laboratory animals This disappears on the inclusion of the vitamin in the diet

3 Only a trace of vitamin B_1 is necessary for the promotion and maintenance of growth and preventing the accumulation of pyruvic acid The larvæ can thus be used for the detection of very small amounts of the vitamin in biological materials

ACKNOWLEDGMENT

Thanks are due to Dr N B Bhatt, Lecturer in Acoustics Indian Institute of Science, Bangalore, for the construction of the photo electric colorimeter

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Ind Jou Med Res 31, 2 October 1943

THE ROLE OF CALCIUM AND VITAMINS IN TUBERCULOSIS

STUDIES ON SERUM CALCIUM IN NORMAL AND TUBERCULOUS SUBJECTS

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[Received for publication, June 14, 1943]

THE steadily increasing volume of literature on arresting the scourge of tuberculosis bears testimony to the interest taken by research workers all over the civilized world in problems relating to different aspects of this disease This is not surprising in view of the fact that tuberculosis is responsible for a very large number of deaths annually besides incapacitating for arduous work a still larger number who happen to escape its more serious consequences

Since calcium is frequently advocated in ameliorating tuberculous conditions it was considered advisable to find out the specific role played by calcium in this disease

In their extensive investigations the early students of tuberculosis discovered the occurrence of calcareous deposits in old pulmonary lesions Baillie (1797) regarded the earthy concretions in tuberculosis as 'a rare appearance of the disease' Boyle (1810) considered 'calcareous phthisis' as a separate incurable form with very few clinical manifestations and he assumed that it may become fatal in cases with an excessive accumulation of calcareous material in the lungs Laennec (1820) found calcareous collections a frequent occurrence in old tuberculosis lesions and in the walls of cavities

The frequent finding of these earthy concretions in healed tuberculous foci, therefore, suggested to the early workers that calcium might be essential for their repair Local and general calcium deficiency was thus to be expected in an acute tuberculous process and Robin (1894), in examining the ash of tuberculous and normal organs, found a diminution of calcium in tuberculous organs This view stimulated considerable research on calcium metabolism as this mineral is one of the chief basic constituents of the body frame work In addition, calcium is known to be of vital importance to the body because of the part it plays in the coagulation of the blood, in the irritability of the heart and in the excitability of the central nervous system In view of these functions, Pottenger (1924) considered the administration of calcium of particular value in ameliorating tuberculous conditions

Since, according to Halverson *et al* (1917), Kramer and Tisdal (1922), Cruickshank (1923) and Georgy (1924), the blood cells are almost, if not entirely, devoid of calcium, it was considered that the amount of calcium in serum would give a measure of the extent of the demineralization which had occurred and of the seriousness of the disease However, although a number of investigators have attempted to estimate the serum calcium of tuberculous subjects, there seems to be a diversity of opinion in their findings Thus, whereas Tephtz (1925), Greisheimer and van Winkle (1927) Brockbank (1927), Dolgopol (1929) and Kaminsky and Davidson (1931) found low calcium values in far-advanced cases showing extensive lesions and little variation in subjects with quiescent lesions, as compared with normal individuals, Halverson *et al* (*loc cit*) and Matz (1925) found either no deviation from the normal or a slight increase in benign and moderate tuberculous cases

In order, therefore to study the role of calcium in tuberculosis, it is first of all necessary to study the variations in the serum calcium as affected by the various stages of the disease

An important preliminary, however, to this is the study of the serum calcium in normal Indian individuals since, as has already been pointed out by Sokhey (1936) and Kehar *et al* (1940) the figures obtained by workers in other countries might not appertain in India owing to differences in topography climate, diet and heredity

Experimental

The serum-calcium estimations were conducted in an unselected group of patients suffering from pulmonary tuberculosis This group included men and women of different ages and of different social and economic status with varying stages of involvement of the disease Specimens of blood were obtained in the morning before any food was ingested Most of the subjects were persons of average means and their ages ranged from 15 to 46 years

In order to obtain normal standards for comparison, a series of serum-calcium estimations was conducted on healthy subjects living under similar conditions

The serum calcium was estimated by Clark and Collip (1925), a modification of Kramer and Tisdal's (1921) method

Results and discussion

Tables I and II show the amount of calcium in the serum of healthy subjects The average value proved to be 10 84 mg per 100 ml serum (min 9 00—max 13 20) in the case of men and 10 43 mg (min 8 00—max 12 40) in the case of women Similar values were obtained by Kehar (1931 unpublished) in a group of 75 men between 16 and 50 years of age

Table I

Serum calcium of healthy men

Number	Date	Name	Age, years	Mg of Ca per 100 ml
1	5-9-39	S A	15	12 00
2	11-9-39	P C	38	10 00
3	10-10-39	H B S	30	9 00
4	10-10-39	C P S	18	9 25
5	10-10-39	C P	28	9 25
6	10-10-39	C S	30	9 00
7	17-10-39	G S	28	11 00
8	18-10-39	R R	17	10 40
9	4-11-39	C L	22	11 25
10	4-11-39	R K	26	12 60
11	4-11-39	B L	52	12 00
12	4-11-39	B L	30	10 30
13	6-12-39	P N	25	13 20
14	6-12-39	P C	20	11 00
15	8-12-39	R A	34	12 40
16	8-12-39	R B S	32	10 90
17	12-12-39	R C	34	10 00
18	28-12-39	A K	24	10 80
19	28-12-39	B M L	18	11 00
20	28-12-39	G S	26	10 80
21	29-12-39	P G N	37	10 80
22	2-1-40	M S	38	11 40
23	5-1-40	B D S	36	10 80
24	14-1-40	H R	20	10 40
25	5-1-40	P L	32	11 00
26	12-1-40	M R	38	10 20
27	12-1-40	A N	39	10 20
		Average		10 84

TABLE II

Serum calcium of healthy women

Number	Date	Name	Age, years	Mg of Ca per 100 ml
1	3-10-39	S D	22	8 00
2	10-10-39	S D	16	11 50
3	17-10-39	B D	20	11 00
4	22-11-39	G S	30	10 00
5	22-11-39	J D	26	10 70
6	23-11-39	G D	30	10 40
7	28-11-39	G D	25	12 40
8	8-12-39	B D	35	10 60
9	26-12-39	N D	38	10 80
10	27-12-39	P	40	10 00
11	27-12-39	H D	18	10 20
12	27-12-39	R D	22	11 00
13	27-12-39	M	22	10 20
14	28-12-39	S R	38	11 00
15	28-12-39	B D	22	10 20
16	28-12-39	C D	28	11 80
17	29-12-39	V	26	10 80
18	29-12-39	B D	24	10 00
19	6-1-40	B D	40	8 80
20	12-1-40	R K	28	9 10
21	12-1-40	S R	38	10 80
22	13-1-40	R K	38	10 20
		AVERAGE		10 43

The normal range of serum calcium as found by some other workers is given in Table III —

TABLE III

Calcium content of normal human blood serum found by other observers

Name of authors	Number of cases	Mg of Ca per 100 ml		
		Min	Max	Average
Rosen and Krasnow (1926)	50	10 7	13 2	11 66
Matz (1925)	50	9 0	12 0	10 28
Roe and Khan (1928)	50	9 0	11 6	10 15
Kaminsky and Davidson (1931)	154	7 52	11 62	9 99
Dolgopol (1929)	16	9 26	11 40	10 08
Denis and Hobson (1923)				10 00
Jenson (1925)				12 46
Howland and Kramer (1921)				10 00
Present workers (1943) Men	27	9 00	13 20	10 84
Present workers (1943) Women	22	8 00	12 40	10 43

In order to find out whether any relationship exists between serum calcium and the severity of the disease, the patients were divided into two groups, viz 'early' and 'advanced' stages of tuberculous infection

TABLE IV

Serum calcium in 'early' and 'advanced' pulmonary tuberculosis

Stage		Number of cases	Serum Ca in mg per 100 ml		
			Min	Max	Average
Early stage	Women	24	6 75	12 60	9 82
	Men	19	8 40	12 60	10 33
Advanced stage	Women	20	5 80	12 40	9 63
	Men	22	7 80	12 60	10 03

It will be observed from Table IV that when the average values for serum calcium of healthy persons, both male and female, are compared with those for early and advanced cases of tuberculosis, the latter are definitely lower as shown by significance tests, but between the two types of cases the difference in the averages is not significant While the onset of tuberculosis seems to reduce serum calcium the available evidence does not suggest that, with the progress of the disease, there is a corresponding fall in the calcium content of the serum

The serum-calcium concentration in the 'active' and 'quiescent' stages of the disease was further examined and it was found that both men and women in the 'active' group showed a lower average than those in the 'quiescent' group These observations compare favourably with those of Kaminsky and Davidson (*loc cit*) and indicate a greater demineralization in active tuberculosis The difference in the serum-calcium level in both the 'active' and 'quiescent' stages of the disease as compared with the healthy individuals is significant in men though not in women

TABLE V

Serum calcium in 'active' and 'quiescent' pulmonary tuberculosis

Stage		Number of cases	Serum Ca in mg per 100 ml		
			Min	Max	Average
Active stage	Men	28	5 80	11 60	9 28
	Women	18	7 80	12 60	9 79
Quiescent stage	Men	26	7 00	12 40	10 19
	Women	23	8 40	12 60	10 33

Several tuberculous patients with a history of hæmoptysis were also examined and the esults given in Table VI were obtained —

TABLE VI

		Number of cases	Serum Ca in mg per 100 ml		
			Min	Max	Average
Cases with a history of hæmoptysis	Men	8	7 20	11 60	9 98
	Women	7	8 00	12 60	10 22
Cases without a history of hæmoptysis	Men	46	5 80	12 60	9 78
	Women	34	7 80	12 60	10 20

It will be seen that although in the case of men there is a decrease in the serum calcium as compared with the average normal calcium values there appears to be little change in the case of women patients The little difference observed in the case of men also is not statistically significant Kaminsky and Davidson (*loc cit*) also failed to find any appreciable variation although they do not mention whether their subjects were male or female

It has been pointed out that if the patients are divided into different groups as —

Healthy	*vs* early	Healthy	*vs* advanced
Healthy	*vs* active	Healthy	*vs* quiescent
Quiescent	*vs* active	Early	*vs* advanced

and the difference in the average calcium content is considered with a probability of 5 per cent or less as the level of significance the variations from the normal are significant in the case of men in all the groups except the last whereas they are not significant in the case of women patients

Summary

The serum calcium of 19 healthy and 275 tuberculous men and women living under identical conditions was estimated It was found that —

1 The average amount of serum calcium in men is 10 84 mg and in women 10 43 mg per 100 ml

2 A significant decrease has been found in the case of men in the early, advanced, active and quiescent stages of the disease as compared with healthy individuals However, between early and advanced cases the differences in the averages for serum calcium are *not significant* in respect of both men and women

3 No significant decrease has been noticed in tuberculous women patients in different stages of the disease as compared with the healthy state

4 Hæmoptysis does not seem to affect the level of serum calcium

Acknowledgments

The authors wish to express their gratitude to Mr S K Talapatra and Mr A K Pal for carrying out some of the analyses

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Ind Jour Med Res 31, 2 October 1943

DISTRIBUTION OF BLOOD GROUPS AMONG DIFFERENT COMMUNITIES IN THE GOVERNMENT MENTAL HOSPITAL MADRAS

BY

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(Received for publication March 29 1943)

The blood of 1 181 patients of the Government Mental Hospital, Madras, was typed The patients belonged mainly to the following 18 communities Mohammedan 161, Brahmin 155, Indian Christian 131 Adidravida 70 Chetty 60 Mudaliar 56, Anglo Indian 59, Asari 37, Baliga 33 Reddy 29, Pillai 26 Naidu 25 Gownder 21 Nadar 16, Nair 56 Thiya 54, Naiker 14 and Kummara 10 The percentage of the blood groups together with the frequencies of O A and B and the biochemical indices are shown in Table I —

TABLE I

Showing the percentage of the blood groups, their frequencies and biochemical indices of the various communities in the Government Mental Hospital, Madras

Name of the community	Number of persons typed	Blood group percentages				Biochemical index	Frequencies	
		O	A	B	AB		*p*	*q*
Mohammedan	161	55 9	21 1	20 0	3 1	1 05	7 476	1 289	1 23
Brahmin	155	45 8	19 4	29 7	5 1	0 7	6 769	1 3075	1 9235
Indian Christian	131	37 4	17 6	38 1	6 9	0 54	6 116	1 305	2 581
Adidravida	70	32 9	17 1	47 1	2 9	0 4	5 736	1 195	3 069
Chetty	60	21 7	23 3	48 3	6 7	0 54	4 659	1 8485	3 4925
Anglo Indian	59	01 5	25 6	10 3	2 6	2 3	7 843	1 508	0 640
Mudaliar	56	33 93	23 21	33 93	8 93	0 75	5 825	1 744	2 431
Nair	56	31 25	25 0	37 5	6 25	0 68	5 591	2 661	1 748
Thiya	54	57 1	35 7	7 2		4 8	7 556	2 0295	0 4145
Asari	37	35 1	27 0	37 9		0 71	5 926	1 7145	2 3595
Baliga	33	45 5	23 2	23 2	6 1	0	7 063	1 4685	1 4685
Reddy	29	62 1	10 3	24 1	3 5	0 5	7 871	0 6765	1 4525
Pillai	26	38 46	15 38	38 46	7 7	0 89	6 201	1 1835	2 6155
Naidu	25	36 0	16 0	48 0		0 33	6 008	1 029	2 7015
Gownder	21	33 3	23 8	42 9		0 55	5 771	1 5275	2 7015
Nadar	16	18 8	25 0	56 2		0 45	4 336	1 8105	3 8535
Naikar	14	78 6	14 3	7 1		1 93	8 865	0 7575	0 3775
Kummara	10	20 0	10 0	50 0	20 0	0 4	4 472	1 3285	4 1995

The frequencies are calculated according to the formulæ employed by Wellisch (1929) and Parr (1931) —

p = frequency of A = $\frac{1}{2}(1 - r + \sqrt{O + A} - \sqrt{O + B})$

q = frequency of B = $\frac{1}{2}(1 - r + \sqrt{O + B} - \sqrt{O + A})$

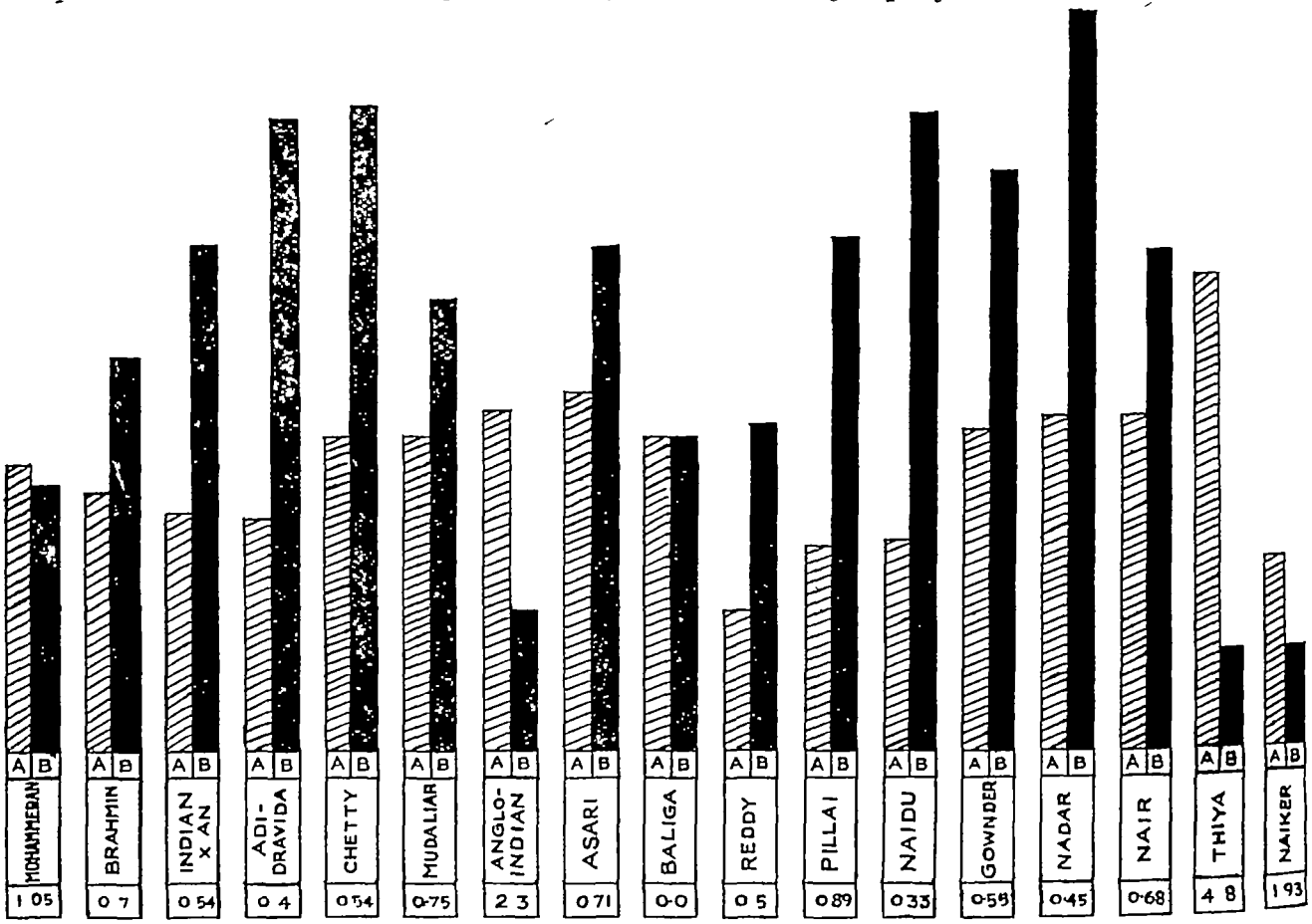
r = frequency of O = $\sqrt{O}$

The biochemical index is determined according to the formula $\frac{A + AB}{B + AB}$

The relation between the percentage of A and B blood groups in the different communities is shown in the Chart —

CHART

Showing the relation between percentages of A and B blood groups of the various communities.



The biochemical index of the Thiyas is very high It is higher than the highest biochemical index of the European types given by Snyder (1926) The biochemical index of the Anglo-Indians approaches the lowest biochemical index of the European types The biochemical indices of the most of the communities fall within the Asio-African type of Kappers and Parr (1934)

A study of the blood groups on a linguistic basis reveals the fact that the biochemical indices of the Telugu people and the Canarese people are similar, while those of the Tamils and Malayalees are almost identical The blood group percentages, frequencies and biochemical indices of the Telugu, Tamil, Canarese and Malayalam speaking peoples are given in Table II —

TABLE II

Showing the percentages of the blood groups, their frequencies and biochemical indices of Telugu, Tamil, Canarese and Malayalam speaking peoples

Name of the community	Number of persons typed	Blood group percentages				Biochemical index	Frequencies		
		O	A	B	AB		*r*	*p*	*q*
Andras	331	48 9	21 7	25 4	4 0	0 86	6 993	1 4	1 607
Tamils	577	39 0	22 9	33 9	4 2	0 72	6 246	1 5415	2 2125
Canarese	82	50 0	21 0	25 8	3 2	0 84	7 071	1 3245	1 6045
Malayalees	191	30 6	30 6	36 9	5 4	0 74	5 533	1 9235	2 5535

In Table III are given the values for the Hindus within the hospital along with the values given by other workers —

TABLE III

Distribution of blood groups among Hindus, Mohammedans, Anglo Indians and Indian Christians in Madras Calcutta and the United Provinces

Name of the community	Total blood samples typed	Blood group				Frequencies (Snyder)			Figures published by
		O	A	B	AB	p	q	r	
Hindus, Mental Hospital, Madras	830	41 81	22 86	31 17	4 16	14 7	20 62	64 68	Reddi (present series)
Hindus, Blood Bank donors, Madras	418	42 83	23 92	30 43	3 83				Reddi (under publication)
Hindus, Madras	1,834	39 2	24 42	30 2	4 84	16 68	20 23	62 61	Seshadrinathan and Timothy (1942)
Hindus, Calcutta	1,302	36 2	21 8	34 6	7 5	16 0	23 8	60 0	Greval and Chandra (1940)
Hindus, United Provinces	2,357	30 2	24 5	37 2	8 1	17 9	26 1	54 9	Malone and Lahiri (1929)
Mohammedans, Mental Hospital, Madras	161	55 9	21 1	20 0	3 1	12 89	12 35	74 76	Reddi (present series)
Mohammedans, Blood Bank donors, Madras	95	42 11	25 26	30 53	2 105				Reddi (under publication)
Mohammedans, Madras	141	31 24	28 4	38 34	2 13	16 59	22 70	55 9	Seshadrinathan and Timothy (1942)
Mohammedans, Calcutta	321	29 5	24 6	36 4	9 3	18 8	26 5	54 3	Greval and Chandra (1940)
Anglo Indians, Mental Hospital, Madras	59	61 5	25 6	10 3	2 6	15 08	6 49	78 43	Reddi (present series)
Anglo Indians, Blood Bank donors, Madras	37	48 65	35 14	13 52	2 703				Reddi (under publication)
Anglo Indians, Madras	47	55 32	19 5	25 32					Seshadrinathan and Timothy (1942)
Anglo Indians, Calcutta	346	37 2	37 8	19 3	5 4	24 8	13 4	60 8	Greval and Chandra (1940)
Anglo Indians, I M D students	67	40 20	41 79	14 92	2 98	25 7	9 4	63 2	Greval and Chandra (1940)
Indian Christians, Mental Hospital, Madras	131	37 4	17 6	38 1	6 9	13 05	25 81	61 16	Reddi (present series)
Indian Christians, Blood Bank donors, Madras	27	40 74	22 22	33 33	3 703				Reddi (under publication)
Indian Christians, Madras	95	41 05	23 15	31 5	4 21				Seshadrinathan and Timothy (1942)

The values given herein do not differ very much from those given by Seshadrinathan and Timothy (1942)

ACKNOWLEDGMENTS

I am greatly indebted to Captain Parasuram and Dr Dhiryan of the Government Mental Hospital Madras, for their kindness in giving me all the necessary facilities during the course of this investigation My thanks are also due to Dr A Aiyappan, Superintendent, Government Museum Madras for his kind interest during the course of this work

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DISTRIBUTION OF BLOOD GROUPS AMONG SOME DONORS TO THE MADRAS BLOOD BANK

WITH A DISCUSSION ON THE RELATIONSHIP BETWEEN NEUROPATHIC CONDITIONS AND BLOOD GROUPS

BY

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(Received for publication April 19 1943)

THE blood of 592 donors to the Madras Blood Bank was grouped Samples of blood were collected from the donors in test-tubes containing 3 c c to 4 c c of 2 5 per cent sodium citrate solution The samples were then brought to the Government Museum Madras, for grouping The method of macroscopic slide agglutination was employed and the sera were supplied by the King Institute, Guindy The donors were composed mainly of Brahmins (132), Nairs (147), Thiyas (113) Mohammedans (95) Anglo Indians (37) and Indian Christians (27) The percentages of the blood groups their frequencies and biochemical indices were calculated as described in my previous paper and the results are set forth in Table I —

TABLE I

Number	Name of the community	Total number typed	Blood group percentages				Biochemical index	Frequencies		
			O	A	B	AB		r	p	q
1	Nairs	147	39 46	21 77	34 69	4 083	0 64	6 32	1 445	2 235
2	Brahmins	132	44 7	20 5	31 1	1 8	0 69	6 7	1 325	1 075
3	Thiyas	113	46 02	31 85	18 58	3 54	1 59	6 78	2 0	1 22
4	Mudaliars	17								
5	Adidravida	5								
6	Chetty	4								
	TOTAL HINDUS	418	42 83	23 92	29 43	3 827	0 83	6 55	1 24	2 21
7	Mohammedans	95	42 11	25 26	30 53	2 105	0 84	6 48	1 61	1 91
8	Indian Christians	27	40 74	22 22	33 33	3 703	0 70	6 4	1 465	2 135
9	Anglo Indians	37	48 65	35 14	13 52	2 703	2 33	6 93	2 175	0 895
10	Europeans	15								
	TOTAL OF ALL PERSONS TYPED	592								

The results for the various communities are in agreement with those of the previous workers The figures for Thiyas and Anglo-Indians confirm the author's previous figures for the same communities These results have to be explained by taking into account the racial crossing that has been taking place for a long time in these communities

Discussion on Neuropathic conditions and blood groups

In a previous paper (Reddi, 1943) the author has given the distribution of blood groups among 1,181 patients of the Government Mental Hospital, Madras The patients were classified according to the case sheets of the hospital under eight neuropathic conditions, i e (1) schizophrenia, (2) dementia præcox, (3) senile psychosis, (4) toxic psychosis, (5) puerperal psychosis, (6) epileptic insanity, (7) mental defective and (8) mania Table II gives the figures for the distribution of the blood groups for the various types of mental diseases —

Table II

Number	Type of neuropathic condition	Total number of persons typed	Blood group percentages				Biochemical index	Frequencies		
			O	A	B	AB		*r*	*p*	*q*
1	Schizophrenia	231	45 9	16 9	34 6	2 6	0 52	6 775	1 0885	2 1365
2	Dementia præcox	27	40 8	22 2	29 6	7 4	0 8	6 389	1 5795	2 0315
3	Mania	247	40 8	25 4	29 8	4 0	0 89	6 311	1 7115	1 9775
4	Mental defective	332	41 3	22 3	31 6	4 8	0 74	6 442	1 498	2 06
5	Epileptic insanity	67	37 3	34 3	25 4	3 0	1 31	6 108	2 2175	1 6745
6	Senile psychosis	93	43 0	22 6	29 0	5 4	0 81	6 711	1 4515	1 8375
7	Puerperal psychosis	121	47 6	19 0	28 6	4 8	0 71	6 9	1 266	1 834
8	Toxic psychosis	63	34 9	27 0	33 3	4 8	0 83	5 907	1 8515	2 2415

The figures for one neuropathic condition do not show any significant deviation from any other type of neuropathic condition Only in the case of epileptic insanity and toxic psychosis we get a high percentage of the blood group A This is not due to any linkage between the particular neuropathic condition and the blood group concerned The patients under these neuropathic conditions are mostly Anglo-Indians and Thiyas who normally exhibit a high percentage of A If we compare the distribution of blood groups of normal Brahmins, Nairs, Thiyas and Anglo-Indians who have donated blood to the Madras Blood Bank with the distribution of the blood groups within the same communities of the Mental Hospital we do not find any significant variation at all as shown in Table III —

Table III

Name of the community	Total number of persons	Blood groups				Frequencies			Figures published by
		O	A	B	AB	*r*	*p*	*q*	
Nairs (insane)	56	31 25	25 0	37 5	6 25	5 59	1 75	2 66	
Nairs (normal)	147	39 46	21 77	34 69	4 08	6 32	1 45	2 24	
Brahmins (insane)	155	45 8	19 4	29 7	5 1	6 76	1 31	1 92	
Brahmins (normal)	132	44 7	20 5	31 1	3 8	6 7	1 32	1 97	
Thiyas (insane)	54	57 1	35 7	7 2		7 5	2 02	0 41	
Thiyas (normal)	113	46 02	31 85	18 58	3 54	6 78	2 0	1 2	
Hindus (insane)	830	41 81	22 86	31 17	4 16	6 47	1 47	2 06	
Hindus (normal)	418	42 83	23 92	29 43	3 83	6 55	1 24	2 21	
Hindus (normal)	1 834	39 2	24 4	30 2	4 84	6 26	1 66	2 02	Seshadrinathan and Timothy (1942)
Hindus (normal)	1,302	36 02	21 8	34 6	7 5	6 0	1 6	2 3	Greval and Chandra (1940)
Hindus (normal)	2,357	30 2	24 5	37 2	8 1	5 5	1 7	2 6	Malone and Lahiri (1929)
Anglo Indians (insane)	59	61 5	25 6	10 3	2 6	7 8	1 5	0 64	
Anglo Indians (normal)	37	48 65	35 14	13 52	2 7	6 9	2 17	0 89	
Anglo Indians (normal)	346	37 2	37 2	19 3	5 4	6 08	2 48	1 34	Greval and Chandra (1940)
Anglo Indians (normal)	67	40 29	41 79	14 79	2 98	6 32	2 57	0 94	Greval and Chandra (1940)

The neuropathic conditions show no linkage with the blood groups

Alexander (1921) stated that the distribution of blood groups B and AB exhibited some association with malignant disease Buchanan and Higley (1921) also disagreed with Alexander, while the work of Pfahler and Widmann (1924) contradicted the results of Alexander

Johannsen (1925) who studied 263 cases of malignant disease maintained that some sort of linkage existed between the disease and O and B blood groups, while Weitzner (1925) studying 84 cases of carcinoma found some association of carcinoma with blood group AB

Hirszfeld Hirszfeld and Brokman (1924) tried to show some association between blood groups and diphtheria Straszynski (1925) associated the rapid disappearance of the Wassermann reaction under treatment with blood groups while Furst (1925) attempted to show the relation of goitre to the blood groups

Snyder (1924 1924*a*) gave a complete review of the literature dealing with the relation between blood groups and anæsthesia drugs roentgen rays and showed the general agreement of the several investigators that the distribution of the blood groups is not affected by age sex, disease drugs anæsthesia etc Snyder (1926) worked on several hundred cases of neuropathic conditions both among white races and American Indians and showed that the distribution of blood groups among them agreed with that for normal persons of the same race

My findings are in entire agreement with those of Snyder (1926)

Acknowledgments

I am greatly indebted to the Director of the King Institute Guindy for having kindly permitted me to take samples of blood from the donors to the Madras Blood Bank My thanks are also due to the Medical Officers who were in charge of the Blood Bank for the facilities they gave me and to the Superintendent of the Madras Government Museum for his kind interest during the course of this work

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ON ISOHÆMOLYSIS REPORTS ON TWO ISOLYSINS AND ASSOCIATED CONSIDERATIONS

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[Received for publication August 10, 1943]

A STRONG ISOLYSIN (ISOHÆMOLYSIN) b ASSOCIATED WITH ISONIN (ISOHÆMAGGLUTININ) b IN A SERUM ab (OF A SUBJECT O)

It was noticed that a fresh serum ab agglutinated within minute cells A very strongly and lysed cells B completely A sample of cells AB was also lysed though not as rapidly as the sample of cells B The isonin (isohæmagglutinin) a was thought to be of a high order The serum was inactivated (at 56°C for 30 minutes) to see if the isonin b was of a higher order Contrary to expectation both the isonins were found to be of the same middling titre

Fuller details of the reactions of the serum are given in the Table The following unexpected and unusual findings are recorded —

1 *Inhibition of hæmagglutination by inert isolysin* —The isolysin when rendered inert for immediate action by dilution or by inactivation of the native complement of the serum was found to inhibit the action of the isonin (*i*) The fresh serum in dilution prevented hæmagglutination until weak dilutions were reached, (*ii*) the partly inactivated serum (56°C for 15 minutes) also prevented full hæmagglutination, and (*iii*) even fully inactivated serum (56°C for 30 minutes) prevented full hæmagglutination in a strong dilution

The inhibition was traceable to the complement which was protected by the isolysin, to some extent, against inactivation as usually carried out When the inactivation was prolonged to 60 minutes the inhibition disappeared

2 *Absorption of complement by barium sulphate* —The intention was to absorb the isonins and leave the isolysin intact, as recommended by Lattes (1932) The opposite effect was seen the serum became less lytic in a 1 in 2 dilution and the agglutination of r b c B was improved in a 1 in 16 dilution Obviously the complement was absorbed

3 *Unusual unstability of granularity near the end of inhibition* —The granularity appearing when the zone of inhibition of hæmagglutination was just passed was unusually unstable It was very easily resolved by agitating the fluid on the slide In a small test-tube it could not be made out at all

4 *Delayed lysis caused by old and diluted serum* —A delayed hæmolysis of the agglutinated r b c also occurred The r b c (now without colour) remained clumped

The technique followed in these and the following experiments, excepting the experiment on re-activation, was of open preparations on slides kept in moist chamber made of Petri-dishes The volumes were measured by calibrated capillary pipettes Details have been published previously (Greval Chandra and Woodhead, 1939, 1941)

Contrary to the senior writer's (S D S G) usual plan of titration the dilutions used in the titration of the serum were 1 in 2 1 in 4 1 in 8, 1 in 16 and 1 in 32

TABLE.

Reactions of Sukhu's serum, ab containing isolysin b.

	Lysis caused by isolysin b in a dilution of 1 in —						Agglutination caused by —											
							Isonin a in a dilution of 1 in —						Isonin b in a dilution of 1 in —					
	1	2	4	8	16	32	1	2	4	8	16	32	1	2	4	8	16	32
Serum, fresh	L	L	T	—	—	—	++	++	++	++	++	+	r b c lysed	—	—	—	±	+
Serum, inactivated by —																		
Ago (taken 16 days previously and kept in refrigerator)	P	T	—	—	—	—	++	++	++	++	++	+	—	+	+	++	++	+
Heating at 56°C for —																		
15 minutes	T	—	—	—	—	—	++	++	++	++	++	+	±	±	±	++	++	+
30 ,,	—	—	—	—	—	—	++	++	++	++	++	+	+(+)	++	++	++	++	+
60 ,,	—	—	—	—	—	—	++	++	++	++	++	+	++	++	++	++	++	+
Serum, absorbed with barium sulphate for 2 hours	L	P	—	—	—	—	++	++	++	++	++	+	r b c lysed	—	—	—	+	+

Dilution of 1 in 1 = undiluted serum
L = Lysis
P = Partial lysis
T = Trace of lysis

++ = Centrifugal clumps
+(+) = Both kinds of clumps
+ = Centripetal clumps
± = Granularity

The suspensions of r b c in sera were made thus about 0 2 c c of the 2 per cent suspensions in saline was put in small tubes and the levels marked, the tubes were centrifuged and the saline discarded and the required sera were added to the deposit of the cells to the marked level

Testing for anti-isolysins

According to the hypothesis of Lattes (*loc cit*) the complete serological constitution of the blood groups would be as follows —

	Group O	Group A	Group B	Group AB
Isogen	0	A	B	AB
Isonin	ab	b	a	o (=nothing small letter)
Isolysin	ab	b	a	o (=nothing, small letter)
Anti isolysin (anti isohemolysin)	o (=nothing small letter)	anti a	anti b	anti a and anti b,

The strong isolysin b was used in detecting the presence of the supposed anti-isolysin b in the blood of four subjects B Their r b c and sera were obtained and put up with the lytic serum in six ways (*i*) 2 per cent r b c suspensions in saline with an equal volume of the lytic serum—result quick lysis (*ii*) 2 per cent r b c suspensions in subjects' own sera with an equal volume of the lytic serum—result delayed lysis in three cases and much delayed lysis in one case, (*iii*) 2 per cent r b c suspensions in absorbed serum a (isonin removed by previous absorption with cells A) with an equal volume of the lytic serum—result, delayed lysis, (*iv*) 2 per cent r b c suspensions in absorbed serum b with an equal volume of the lytic serum—result, delayed lysis, (*v*) 2 per cent r b c suspensions in absorbed serum ab with an equal volume of the lytic serum—result, delayed lysis, and (*vi*) 2 per cent r b c suspensions in serum o, from subject AB, with an equal volume of the lytic serum—result, delayed lysis

The subjects' own sera protected the cells Even human sera from other groups protected the cells The protection at its best, however, was only slight The protecting substance was not more than could be expected to exist in the serum as a result of solution from the cells

The isogens A and B are known to be diffused in the body fluid The senior writer has pointed out that because of the presence of the isogens in the serum the agglutinating titre of a mixture of serum a and serum b is below the calculated figure (Greval, Chandra and Woodhead, 1941) The status of the anti-lysins in serology would, therefore, correspond to that of the aggressions in bacteriology The aggressions are according to most workers broken bits of bacteria in solution

Lattes accepts the explanation of the isogens in solution for the neutralization of the isonins but not for the neutralization of the isolysins which according to him are always opposed by anti-isolysins

Re-activation of the isolysin

The opinion on re-activation is divided some workers (Bezzola, Ascoli, Moreschi and Micheli, quoted by Lattes, *loc cit*) have not been able to re-activate a heated serum, others (Moss, Grafe, Grahm, Schiff and Adelsberger, quoted by the same authority) were nearly always able to do so when the serum was heated to 50°C to 55°C for 15 to 20 minutes but not to 56°C for 30 minutes, and yet others (Thomsen and Thisted, quoted by Wiener, 1935) appear to be regularly successful regardless of the degree and duration of heating

The present writers were able to re-activate the serum after it had been heated to 56°C for 15 minutes and 30 minutes but not for 60 minutes The hæmolysis, however, although complete, did not occur immediately Even the serum partly inactivated by age and re-activated by fresh complement could not hæmolyse the r b c immediately

Fresh complement from subject O acted before the one from subject AB Guinea-pig complement acted last

The mixtures were made thus (*i*) inactive serum one volume *plus* the added complement (from O, AB or guinea-pig) one volume, and (*ii*) inactive serum one volume *plus* the added complement three volumes These mixtures were put up with equal volumes of a 2 per cent suspension of r b c B in small conical tubes Hæmolysis commenced at room temperature and was complete in the incubator under 20 minutes with human complement and under 30 minutes with guinea-pig complement

The fresh serum *diluted with saline* actually produced less hæmolysis than the inactive serum diluted with complement, showing that the isolysin content did not represent an excess such as is used in determining the MHD of the complement

Absorption of the isonin and the lysin

The serum was chilled and left in contact with chilled r b c B, for one hour, in a refrigerator to absorb the isonin The tube containing the serum and the cells was quickly transferred to a padded and cooled container and centrifuged The slightly pink fluid, on coming into contact with fresh r b c B at room temperature, did not agglutinate the cells or deepen in colour by lysing them The isolysin had disappeared with the isonin

The serum was left at room temperature and packed r b c B added until no further lysis occurred The red fluid was cleared of debris by centrifuging and tested for agglutination None occurred The isonin had disappeared with the isolysin

The serum was made milky with barium sulphate and left at room temperature for one hour and in the refrigerator for one hour and centrifuged The effect of the isolysin was slightly reduced through partial inactivation

A weak isolysin a associated with isonin a in a serum ab

It was known that the isonin a of a subject O (S D S G) was stronger than the isonin b In view of the partial inhibition of the action of the isonin by the associated lysin, the presence of isolysin b was suspected Fresh serum was put up with r b c A and B Contrary to expectation a weak isolysin a was found lysing the r b c A The isolysin was missed previously because the serum was tested a day after it had been taken

Certain differences were found between the strong and the weak isolysin The weak isolysin did not inhibit the action of the associated strong isonin Its lytic action weakened appreciably in 4 hours The next day it was found completely inert but could be re-activated by adding excess of fresh human complement from a subject AB (one volume aged serum *plus* three volumes of complement *plus* r b c suspensions yielded lysis) Specimens inactivated by heat could not be re-activated

Isolysins in the blood of 'universal' donors

The senior writer is against accepting subjects O with high-titre isonins as universal donors (Grewal Chandra and Woodhead, 1941) From his list of 'safe' universal donors for the blood transfusion service for hospitals in Calcutta he also excluded group O subjects with isolysins The fact, though mentioned, was not stressed It is stressed now The same precaution applies to safe donors A and B for recipients AB

Resemblances and differences between a hæmolytic amboceptor, an isolysin and an isonin, and associated considerations

An artificially produced hæmolytic amboceptor, in a strong dilution, agglutinates the appropriate r b c in the absence of the complement and lyses the same r b c even in a weak dilution in the presence of the complement It reacts readily with the added complement An isolysin as such has no agglutinating action but lyses the appropriate r b c in the presence of the complement with which it is closely linked in the serum It also reacts with the added

complement though to a limited degree An isonin only agglutinates the appropriate r b c and its action is independent of the complement

The link between the original complement and the isolysin is stronger than the one between the amboceptor and the complement of the serum in which it occurs The isolysin protects the original complement against inactivation to some extent

The fact that isolysins are usually associated with high-titre isonins creates an impression that the isohæmolysis is a function of a strong isonin Observations of workers on the 'accidents caused by 'dangerous universal donors have not made this point clear The accidents are caused by at least two processes (*a*) isohæmolysis and (*b*) iso hæmagglutination High-titre isonins have been held responsible for both of them in a general way The *rapid and complete* hæmolysis occurring *in vitro* between incompatible bloods is definitely caused by the isolysin alone A *slow and partial* hæmolysis from strong agglutination in absorption experiments is also known it occurs in the absence of the complement from the squeeze of the agglutination Re activated isolysins also cause delayed hæmolysis

The association between isolysins and high titre isonins is not absolute The isolysins under report were associated with isonins of a middling titre They can even be demonstrated in the new born (Halban and Jones quoted by Lattes *loc cit*) The majority of isonin-containing sera (from all groups except AB) have no isolysin The latter occur only in about 30 per cent of such sera (Wiener *loc cit*)

The isolysins and isonins are contained in the same substance They may even be two phases of the same antibody Inhibition of the action of the isonin by the isolysin is contrary enough an action to be comparable to hypersensitiveness preceding immunity in the development of resistance, in immunology The isolysins (found in the new born whose isonins are weak or absent) may be forerunners of isonins Subjects having both the antibodies could be tested at different ages for the relative titre of each A fall in the isolysin accompanied by a rise in the isonin would point to the soundness of this hypothesis

SUMMARY

1 A strong isolysin b was found in a subject O with isonins of equal and middling titre No anti-isolysins in four subjects B could be detected by its use It could be re-activated It could not be separated from the associated isonin It inhibited hæmagglutination

2 A weak isolysin a was found in another subject O with isonins of unequal and middling titre It did not inhibit hæmagglutination Its re-activation was more limited

3 Isolysins should disqualify universal donors and donors A and B for subjects AB

4 Artificially produced haemolytic amboceptor, isonins and isolysins are compared Attention is drawn to lack of clarity in observations making high-titre isonins responsible for all accidents caused by dangerous universal donors

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Three slides are prepared from one serum On the two sides of each slide the agglutination should be simultaneous and equal If not the serum is rejected

The sera not rejected are titrated further according to the scheme of titration previously described (Greval, Chandra and Woodhead, 1941) Those showing inequality on dilution are rejected and others titrated further until the last effective dilution is reached This dilution gives the *Minimal Dose of Equal and Simultaneous Agglutination*, the MDESA (used for singular and plural alike) The only latitude allowed is that one side may begin to show agglutination a fraction of a minute earlier but it must look the same as the other side after 30 minutes (recorded 'a then b' or 'b then a') The dose selected for absorption is three times this dose Obviously, a serum which does not act in a 1 in 3 dilution is useless

The suspensions of r b c are divided into three lots one lot is used in the titration one in the test next day and one acts as a reserve for repetition if necessary

Care is taken to exclude slow A's' (Greval *et al loc cit*)

The stain-bearing material or dried blood —It must be established at the outset that the specimen consists of human blood and that no animal blood (from easily available mammals and birds) is present The material if moist is exposed to the room temperature and allowed to dry Twenty-five mg of it are cut up into small strips about 1 mm broad and introduced into a small conical tube (*see* Plate III—these tubes are made in the laboratory from glass-tubing) fitted with a small cork (obtained from suppliers of glassware etc to perfumers), suitably marked or labelled Of dried blood only 10 mg are taken

The controls —Five controls are put up —

1 The blank, a control from the unstained portion of the cloth, etc An area of the same size as the stain (or 20 mg) is cut up and put in a conical tube Of surfaces (wood plaster, etc) an equal unstained area is scraped to the same depth as the stained area has been scraped and put into the tube When only scrapings are received the object scraped must be obtained and half the weight of the scrapings scraped from the unstained area for the blank scraped blood is mostly blood, if it is blood at all, and does not contain much of the scraped object From surfaces which cannot be scraped (glass, china, metal, etc) an equal area is marked off by a circle drawn with a match dipped in melted hard paraffin The area is washed in 3 or 4 drops of saline dropped on it and sucked back by a pipette The washings are put into the tube and allowed to dry It should be noticed of course, if the stain is on a clean surface or superimposed on a surface already stained by some other material a control of this material will also be necessary if the stain is superimposed A fabric which must not be cut up is also washed like a surface after it has been stiffened by paraffin applied to the reverse

2 A known stain of group A, marked cont A

3 A known stain of group B, marked cont B

4 A known stain of group O marked cont O

5 Serum-control marked serum cont, an empty tube for receiving the same dilution of the serum with which the tubes of the controls and the test proper are going to be charged

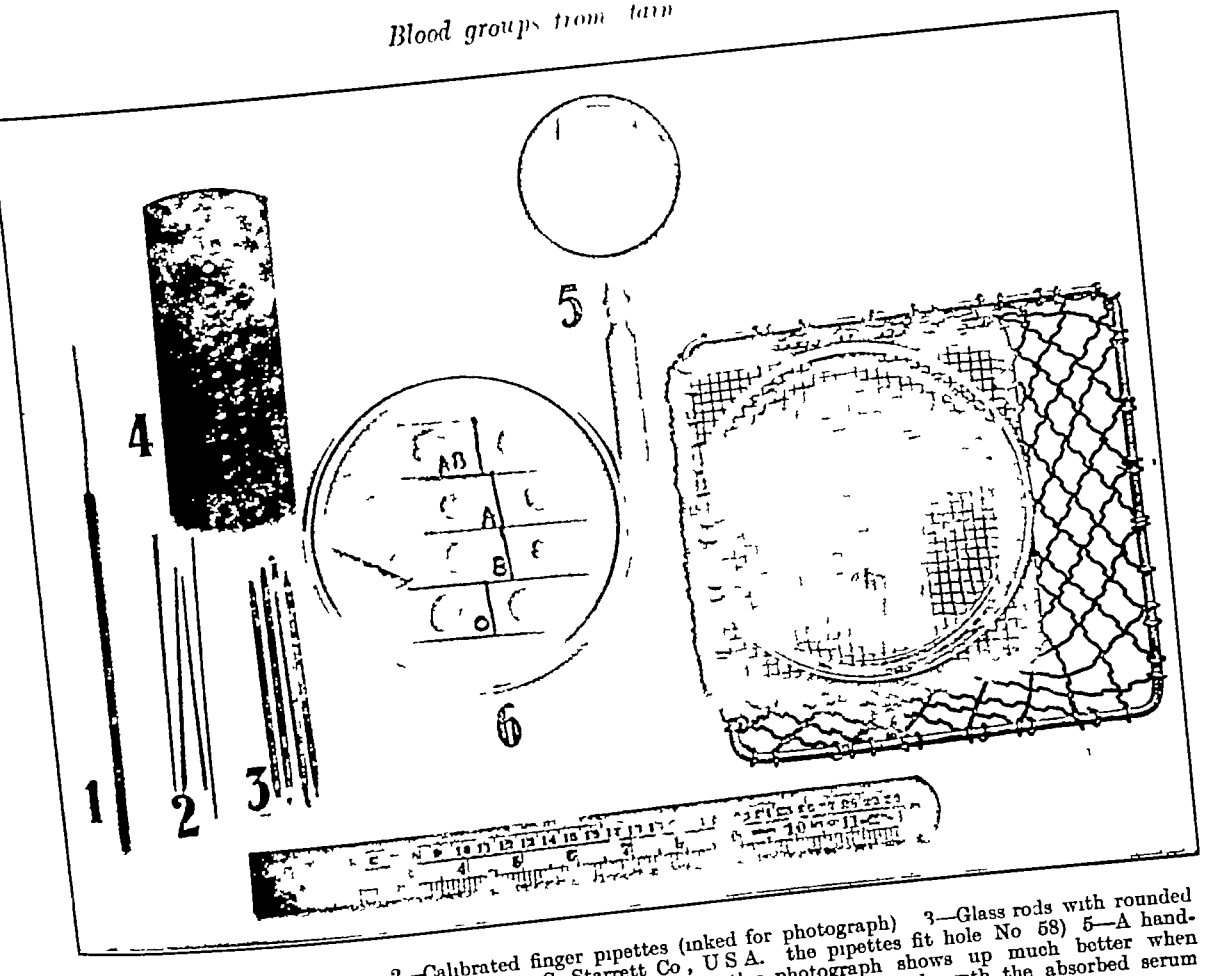
The known controls are of the same age or older than the stains under investigation They are obtained from known donors of blood

The contact with serum ab —Five drops from the standard capillary pipettes, totalling 0 1 c c of the diluted serum containing 3 MDESA in a volume are dropped into the tubes With a stout platinum wire, suitably mounted, the serum and the material are rubbed together until no free fluid is left The dry blood is rubbed into a paste

The tubes are corked fixed sub-horizontally into Petri-dishes by plasticine and incubated at 37°C for half an hour The Petri-dishes are then put in wire-cages which are sealed and left in a refrigerator overnight The photograph shows Petri-dishes in a cage For sealing, the cage is placed on a piece of muslin the edges of which are drawn together to improvise a bag The neck of the bag is sealed

PLATE III

Blood groups from farm



1—A stout platinum wire 2—Calibrated finger pipettes (inked for photograph) 3—Glass rods with rounded ends (inked for photograph) 4—Wire gauge (L S Starrett Co, U S A. the pipettes fit hole No 58) 5—A hand-lens to examine the agglutination critically (the agglutination in this photograph shows up much better when examined with a hand lens like this) 6—A moist chamber containing preparations made with the absorbed serum ab 7—A wire cage for Petri-dishes containing charged specimen tubes (note the corks and sub horizontal lie)

A 12 inch measure gives the actual sizes

Separation of the serum after contact —Next morning the contents of the tubes are packed tight with the stout platinum wire unless they are of the nature of sawdust which does not stay packed The wire is washed between two packings The tubes are centrifuged A clear fluid becomes visible mostly on a deposit sometimes under a serum Drops are removed by calibrated capillary finger pipettes (one for each tube) for testing Packing to centrifuging takes half an hour If not extra time is allowed at room temperature to eliminate the activity of any cold agglutinins present

The test of the r b c suspensions to be used in a day's work —The suspensions kept for the purpose after the determination of the MDESA are tested for auto agglutination A drop from each suspension is added to a drop of saline and stirred in the usual way There should be no auto agglutination in 30 minutes Any change in agglutinability with the serum will be detected in the second serum control (*vide infra*)

The test of the controls

1 *The serum control* —Two drops of the dilution which has been incubated and left in the refrigerator are tested Equality simultaneity and speed should be unimpaired This reaction is to be compared with that of (*i*) known O control and (*ii*) blank

The serum is diluted with 2 volumes of saline It now contains 1 MDESA in a volume It is tested again for equality and simultaneity in 5 minutes There should be no loss of these qualities Loss sometimes occurs and is *indicative of a change in the r b c suspension* rather than in the serum It is not difficult to fit in another suspension of r b c (giving the same MDESA)

It will be observed that *two* slides are prepared and tested from the serum control

2 *The known O control* —With the fluid obtained from this tube the agglutination should occur almost as it occurs with the serum dilution containing 3 MDESA in a volume Commencement may be delayed for a minute or so even simultaneity may be affected for a fraction of a minute but the ultimate equality and intensity should be unimpaired

The four slides prepared so far (No 1 for testing auto agglutination of r b c No 2 serum control strong No 3 serum control weak and No 4 known O control) are left in the same Petri-dish, in fact they are prepared together The r b c suspensions are added on the two halves of the slides as single operations The time is noted after the suspensions have been stirred One stirring-rod stirs all the four slides thus firstly slide for auto-agglutination secondly weak serum control thirdly strong control and fourthly known O control

3 *The known A control* —The fluid after absorption will either agglutinate r b c B alone or give a ± reaction with r b c A also, due to some isonin a left unabsorbed by the known stain The unabsorbed isonin is made ineffective by dilution Three drops of saline (measured by the calibrated pipette) are added to the remaining three drops of the fluid in the tube The original finger pipette still in the tube is emptied and removed to an empty tube Mixing and centrifuging are done again The same finger pipette is put in and two drops removed for the test again The ± reaction is almost certain to disappear leaving the other reaction intact

Even a second dilution with two drops of saline may be undertaken at times

The first dilution reduces the content of the fluid to 1½ MDESA on the unabsorbed side and the second to 1 MDESA

With the known controls which provide sufficient blood for the complete absorption of the appropriate isonin dilution should not be necessary as a rule

4 *The known B control* —The fluid after absorption will either agglutinate r b c A only or give a ± reaction with r b c B also The unabsorbed isonin b is eliminated by dilution as isonin a is in the case of the known stain A

5 *The blank* —The fluid from this tube should not show either a marked decrease in its agglutinating power or any loss of equality and simultaneity, in five minutes If it does, the

tube corresponding to it in the test proper is rejected Substances other than blood interfering with absorption are present The specimen is not suitable for the determination of the group

The test proper

Stains not imparting any colour to the clear fluid in the centrifuged tubes are rejected They are insoluble and their power of absorption has also decreased The writers are aware that blood groups have been determined from tissues from mummies the precaution, however is considered necessary in medico-legal work in view of the difficulties they have experienced in the case of some insoluble stains of known blood When colour is discernible drops are tested with r b c A and B The following reactions and readings will result —

1 Both suspensions are agglutinated sharply equally, simultaneously and almost immediately The blood under investigation is O The reaction should be like the one given by the strong dilution of the serum control Time limit—under 5 minutes

2 Neither suspension is agglutinated The blood under investigation is AB Observation is extended to 30 minutes ± reactions are eliminated by dilution, especially if dilution has been found necessary in the controls

3 Suspension A is agglutinated frankly (if not sharply) and suspension B not at all The blood under investigation is B Observation is extended to 30 minutes Dilution is employed if necessary If during dilution the agglutination of suspension A loses its frankness and becomes ±, opinion is withheld

4 Suspension B is agglutinated frankly (if not sharply) and suspension A not at all The blood under investigation is A Observation is extended to 30 minutes Dilution is employed if necessary If during dilution the agglutination of suspension B loses its frankness and becomes ±, opinion is withheld

It will be observed that the hæmagglutination referred to under 1 is 'sharp' and under 3 and 4 '*frank, if not sharp*' Such is often the case All hæmagglutination however, may be sharp, as is shown in the photograph

Remarks on the technique and associated considerations

From the account so far given details have been withheld with a view to avoiding digression They will now follow —

Weight of the dried blood in a stain —Accurate weighment of the blood contained in a stain is not necessary In the early part of this work such a weighment was made Equal areas of stained and unstained portions of the material (pieces of cloth or filter-paper) were dried and weighed The differences in weight gave the weight of the dried blood in the stain Soon it was found that the isogens may so differ with respect to their capacity of absorbing the appropriate isonins as to nullify the effect of an accurate weighment Further, there is reason to believe that excessive desiccation interferes with the solubility and power of absorption of the stain

For the purpose of this work stains from bloods of known group were obtained by drying a few drops of whole blood dropped on pieces of white drill of medium weight, cut from clean but old overalls and aprons Measured volume of blood was also similarly dried The relations between the volume of the whole blood weight of its stain and weight of the dried blood in the stain were of the following order —

(*a*) 0 5 c c of whole blood = 572 mg of stain (on drill) = 155 mg of dried blood in the stain

25 mg of stain (used in the test), therefore contain equivalent of $\frac{25 \times 0\,5}{572} \times \frac{12\,5}{572} = 0\,02$ (approx) c c of whole blood

(*b*) 10 mg of dried blood scraped from objects (used in the test) contain equivalent of $\frac{10 \times 0\,5}{155} = \frac{5}{155} = \frac{1}{31} = 0\,03$ (approx) c c of whole blood

The stains received in this laboratory in connection with medico legal work are mostly on pieces of cotton fabric The quantity of dry blood in them in a given weight, approximates fairly closely to the quantity in the stain used for experimental work A thick course fabric weighs more than a thin fabric but also holds more blood in the interstices From a tough non absorbing fabric enough blood can be scraped

Preservation of the testing serum —The serum keeps in a refrigerator for several weeks Once a suitable subject has been found large quantities can be collected phenolized (0 25 per cent) and kept

A suitable serum will be found to work with several specimens of appropriate r b c , so that the same serum can be used with different lots of r b c collected on different days It is essential however to confirm the MDES of the serum with a new lot of the r b c intended for use in the test

Retardation of agglutination

(*i*) *When the serum after absorption agglutinates both suspensions* —The reaction is only interpreted when the speed of the agglutination its equality and its intensity are identical with those of the serum control containing 3 MDES Such a reaction is given by the serum after absorption with stains of group O so characteristically that the writers feel justified in ruling out the identification of group O in the absence of the reaction

Some sort of semblance of equality of agglutination of the two suspensions may be found after a contact of 30 or even 15 minutes with a ab serum which has been absorbed with other groups also It is due to incomplete absorption Reaction of group O therefore, must be read within 5 minutes

(*ii*) *When the serum after absorption agglutinates one suspension only* —More often than not the r b c will not be agglutinated with the same speed and intensity as are seen in the case of the stronger of the two serum controls The agglutination will begin more slowly and will usually be frank not sharp With the absorption of the appropriate isonin some loss of the non appropriate isonin also occurs According to Wiener (*loc cit*) this loss is either non specific or indicative of a partial binding together of the two isonins The writers add the observation that at least in an evenly balanced ab serum the loss does not depend on the serum but on the stain In a batch of absorption experiments with the same serum some A and B stains will absorb non-specifically b and a respectively, others will not That is why frank agglutinations are insisted on In such agglutinations the non-specific process has stayed in the background

(*iii*) *When the serum after absorption does not agglutinate either suspension frankly* —A semblance of double agglutination may be present as a ± agglutination in both the suspensions Agglutination on one side may even become almost frank after some time On dilution the frank reaction disappears On such a reaction opinion is withheld That it has not resulted from content of the serum with stain of group O is evidenced by the delay in appearance of the reaction and the lack of intensity and equality of agglutination It may be caused by absorption with a weak isogen combined with a non-specific absorption of the non-appropriate isonin It may also be caused by absorption with isogens AB (of group AB) both of which are weak and one is weaker than the other

Advantages of the absorption test over the extraction and demonstration of the isonins from stains

(*i*) The isoagglutinins are not found in the extract in a satisfactory titre The writers failed to demonstrate them in most stains

(*ii*) The group of the blood in the stain may be 'defective' The isoagglutinins which can co exist in the group compatibly with the isoagglutinogens may be lacking It must, however, be added that in over 2,000 cases grouped for clinical purposes, in connection with the local blood transfusion service, in this laboratory during the last 14 years (written in 1938) a defective group has never been found

False results and negative results

Occasionally the writers have failed to obtain reactions of the desired distinctiveness from known stains and have withheld opinion, that is, they have obtained negative results They have, however, never obtained false results This feature of the test depends on the fact that in the technique all borderline reactions are eliminated by dilutions and then excluded from consideration

In testing the actual exhibits failures to obtain results are very frequent Firstly, some of the controls from the unstained material (blank) interfere with the isonins, equally or unequally the specimens corresponding to the controls are then not proceeded with Secondly, in the reaction of some of the specimens proceeded with a lack of distinctiveness occurs these specimens are discarded Thirdly the main specimen with which other specimens in a case are to be compared is sometimes among those which have been discarded the other specimens are not then proceeded with

Rejection of a stain when the blank has shown absorption partial or complete, of one or both isonins

Boyd and Boyd (1937) have quoted several workers who have pointed out that 'in a surprisingly large number of cases this unstained material will contain one or more of the blood group receptors, presumably from sweat, urine, animal material, etc , and consequently a positive test for this receptor in the stained portion would mean nothing If only one receptor is found in the unstained material tests may be carried out to detect the possible presence of the other, though it will readily be appreciated that in such a case we cannot expect to establish with certainty the full and exact group of the stain' The present writers add the observation that the comparative concentration of the sweat, etc , in the stained and the unstained material cannot be judged The stain bearing area may have in it more sweat, etc , than the unstained area and may show, consequently, more absorption Deductions from a comparative absorption in this case will be misleading The very existence of such a possibility, if explained fully, will create in the minds of the jury a reasonable doubt as to the interpretation of the test The writers, therefore, reject all specimens the blanks of which have absorbed the isonins

Technique for smaller quantities and for stains other than those of blood

(*a*) *Smaller quantities* —The quantities of the stain and the serum dilution used in the absorption test are 25 mg of the former and 5 drops, from a specially standardized pipette, of the latter But 20 mg and 4 drops or 15 mg and 3 drops will suffice, provided that pipettes with thinner ends are used in removing the fluid from the tube after absorption and in adding drops of r b c suspensions Dilution will be difficult

Similarly quantities of the dried blood scraped from exhibits and the serum dilution can be reduced from 10 mg of the former and 5 drops of the latter to 8 mg , 6 mg or even 4 mg of the former and 4 drops, 3 drops or even 2 drops of the latter

Very small stains are treated by washing with 5 to 6 drops of the serum dilution containing 2 MDESA in a volume The dilution is held in contact with the stains in a teat pipette By squeezing out and sucking back the dilution a reasonably coloured and turbid fluid is soon obtained The fluid is incubated for half an hour at 37°C and removed to a refrigerator for the night Next morning it is treated like the ordinary specimen

To check the spread of the dilution from the stains melted hard paraffin may be used Stains on a hard surface are ringed Soft fabrics are stiffened by an application to the reverse

The writers have obtained *indications* of groups from signatures, on paper, in blood, of a certain fraternity A positive report for forensic purposes, however, could not be given on mere indications

(*iii*) *Stains other than those of blood* —They may also be dealt with in the same way as blood stains, *remembering that all fluids from the body may not in every case have the group-specific substance* and that contact with human faeces destroys group specific substances

A macro technique versus a micro technique

The writers prefer the former to the latter Manipulation of slides, repetitions of observation and discrimination between a mere sedimentation of r b c and agglutination are all easier in the macro- than in the micro technique Further, experience in microscopy is not needed the results can be read even by a jury if necessary

SUMMARY

1 A technique for determining blood groups from stains is described The *Minimal Dose of Equal and Simultaneous Agglutination* (MDESA) of an equally balanced serum ab is determined Three such doses contained in 0 1 c c dilution of the serum are left in contact with 25 mg of stained material or 10 mg of dried blood After incubation and prolonged contact in the refrigerator the serum dilution is separated and tested for loss of isohæmagglutinins (isoins) Only clear negative and frank positive reactions are accepted All doubtful cases are excluded from consideration

2 Remarks on the technique include (*i*) weight of the dried blood in a stain, (*ii*) preservation of testing sera, (*iii*) retardation of agglutination in the final test, (*iv*) advantages of the absorption test over the extraction and demonstration of isoins, (*v*) false results and negative results (*vi*) rejection of a stain when the unstained control has absorbed isoins, (*vii*) technique for smaller quantities and for stains other than those of blood and (*viii*) a macro-technique *versus* a micro technique

3 A photograph gives the essential apparatus and the characteristic macroscopic appearance of the reaction

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STANDARD FOR PAPAIN AND ITS PREPARATIONS

BY

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[Received for publication May 18, 1943]

PAPAIN or papayotin is a proteolytic enzyme found in the fruit or milky juice of the melon tree *Carica papaya* whose digestive action on proteins has been long known The active enzyme can be obtained by pricking the fruits or extracting them in water or through an incision in the trunk of the plant In the presence of air the juice hardens and this hardened product is ground up to a powder and dissolved in water The extract is filtered and the filtrate precipitated with ten volumes of alcohol The resulting precipitate is re dissolved in water, re precipitated with alcohol and then dried *in vacuo* Papain is employed to assist protein digestion in chronic dyspepsia gastric fermentation and gastritis

Although this enzyme is in fairly constant demand it is not included in the British Pharmacopœia probably because other enzymes, such as pepsin and pancreatin which are more or less similar in action, have been included The importance of this enzyme has, however been recognized by inclusion in the British Pharmaceutical Codex 1936 The B P C standard for the proteolytic activity of papain is as follows —

'The amino acids produced by one gramme of papain in the assay process (described in B P C under the heading "Assay") require for neutralization not less than 20 c c of N/10 sodium carbonate'

The assay process described in the B P C does not appear to be quite accurate The substrate casein and the enzyme papain are incubated together in the presence of an excess of N/10 sodium carbonate The pH of this reaction mixture when tested electrometrically with a glass electrode was found to be 9 2 This pH definitely interferes with the digestive action of papain, although the activity of the enzyme may not be completely destroyed In testing any enzyme for its action, it is always desirable to evaluate its activity at its optimum pH Papain exhibits optimal activity at pH 5 0 to 7 0 (Fabre and Frossard, 1925) which evidently harmonizes with the iso-electric points of most protein substrates The pH of 9 2 at which the substrate and the enzyme are incubated according to the method of assay described in the B P C, is far removed from the optimum pH for papain and the activity thus determined will not be a true measure of the potency of the enzyme

In carrying out the routine work of this laboratory, the author had occasion to test several samples of papain by the method outlined in the British Pharmaceutical Codex Almost all the samples failed to conform to the standard (*see* Table I), although the proteolytic activity of most of them when tested under conditions optimum to the action of this enzyme was found to be very good It was therefore apparent that the method of assay' required modification in order to ascertain the true activity of the enzyme As the prescribed 'standard' was based on the method of assay the standard also required alteration In order to suggest a new standard a number of commercial samples of papain, both indigenous and imported available in the Indian market were tested In addition to this, samples of papain freshly prepared from the fresh juice of *Carica papaya* were also tested for their proteolytic activity The results of all these determinations have been taken into consideration in suggesting the standard

EXPERIMENTAL

1 *Assay of commercial samples of papain by the B P C method*—Table I shows the activity of the samples of papain assayed according to the B P C method the activity being calculated for one gramme of papain

TABLE I

Number	Activity in terms of c c of N/10 $NaCO_3$
1	*Nil*
2	4 8
3	2 6
4	9 0
5	10 4
6	12 0
7	12 0
8	14 0
9	13 0
10	12 0
11	11 6
12	9 6

It is evident from Table I that none of the samples conform to the requirements of the B P C standard, as the values are all below 20 c c which is the minimum prescribed

2 *Method of assay* —Gelatine was employed in place of soluble casein as the substrate A 4 per cent solution of gelatine was prepared and the pH of this solution adjusted to 5 0 by the addition of a few cubic centimetres of N/10 NaOH To 50 c c of this solution, 10 c c of citrate buffer of pH 5 0 was added One gramme of the sample of papain accurately weighed was triturated with a few cubic centimetres of water and suspension made up to 20 c c after adjusting the pH to 5 0 Ten c c of this suspension were added to the gelatine-buffer solution Immediately after addition, 25 c c from this mixture were withdrawn and 20 c c of formaldehyde solution freshly neutralized to phenolphthalein with N/10 sodium hydroxide were added and titrated against N/10 sodium hydroxide using phenolphthalein as the indicator The remainder of the mixture was incubated at a temperature of 37°C for a period of 3 hours At the end of this period 25 c c of the reaction mixture were removed and 20 c c of formaldehyde solution previously neutralized to phenolphthalein were added and titrated against N/10 sodium hydroxide to the same end-point as in the previous titration The difference in the two titrations gives the number of c c of N/10 sodium hydroxide neutralized by the amino acids formed by 0 18 gramme of papain acting on 0 714 gramme of gelatine at pH 5 0 This figure is a measure of the proteolytic activity of the sample

The same samples of papain previously tested by the B P C method have been assayed for their activity by the above method and the results are given in Table II —

TABLE II

Activity of commercial samples of papain

Number	Imported to or manufactured in India	Activity in terms of c c of N/10 sodium hydroxide (For 0 18 g of papain in the experiment)	Activity calculated in c c for 1 g of papain	Percentage of digestion
1	Foreign	*Nil*	*Nil*	*Nil*
2	,,	2 5	13 75	30 6
3	,,	1 5	8 25	18 4
4	,,	4 0	22 0	49 0
5	,,	4 5	24 75	55 0
6	Indian	6 6	36 30	80 9
7	,,	6 7	37 05	82 1
8	,,	8 0	44 0	93 8
9	,,	7 0	38 5	85 8
10	dried and powdered juice of *Carica papaya*	6 5	35 75	79 7
11	,,	6 2	34 10	76 0
12	,,	5 9	32 45	72 3

As papain is liable to deteriorate rapidly it was considered of interest to study the rate of deterioration of commercial samples of papain when kept in the laboratory at an average temperature of about 20°C to 25°C for periods of 3 to 6 months In some preliminary experiments it was found that the rate of deterioration varied in different samples It was thought that the grade of purity of the samples might be responsible for this difference in the rate of deterioration A study of the rate of deterioration of samples of papain of various grades of purity has therefore been made and the results are presented in Table III —

TABLE III

Deterioration of papain samples of various grades of purity kept in the laboratory for periods of 3 to 6 months at an average temperature of 25°C

Description of the samples of papain	Activity in terms of c c within a few days of manufacture	Activity in c c after 3 months	Percentage loss	Activity in c c after 6 months	Percentage loss
Dried and powdered juice of *Carica papaya*	6 5	6 1	6	5 8	10 7
Crude papain after one precipitation	8 0	6 9	13 7	6 0	25
Soluble papain (purer than the previous preparation) obtained after two precipitations with alcohol	8 0	6 4	20	5 5	31

3 *Papain preparations* —The B P C mentions the three following preparations of papain —

1 Elixir papain —Each fluid drachm contains 3 grammes of papain with alcohol (90 per cent), distilled water and aromatic elixir

2 Glycerinum papain —Papain 9 per cent w/v with dilute hydrochloric acid, simple elixir and glycerine

3 Liquor-papain-*et*-iridine —Each fluid drachm contains one gramme each of papain, and extract of Iris with glycerine alcohol (90 per cent) and chloroform water

No standards are fixed for these preparations, but the concentration of papain in each preparation is stated in the B P C Employing the same method of assay described above, the amount of each papain preparation to be added to the gelatine solution can be calculated so that the same amount of the enzyme is added to the substrate-buffer solution If this is done, the standard for these preparations will remain the same as for papain itself Instead of adding 10 c c of a 5 per cent suspension of the enzyme the amounts of each preparation to be added will be different, but the concentration of the enzyme in the reaction mixture will be the same as in the assay for the enzyme itself The amounts of each papain preparation to be added, are calculated and given below —

1 Elixir papain —5 c c of this preparation are made up to 10 c c with water after adjusting the pH to 5 10 c c of this solution are added to the gelatine-buffer solution

2 Glycerinum papain —5 6 c c of this preparation are made up to 10 c c with water after adjusting the pH to 5 10 c c of this solution are added to the gelatine-buffer solution

3 Liquor-papain-*et*-iridine —13 5 c c adjusted to pH 5 are added to the gelatine solution In this case the volume of the reaction mixture becomes 73 5 c c instead of 70 c c as in all other cases In order to keep the volume at 70 c c , the amount of buffer solution can be reduced to 6 5 c c , instead of the usual 10 c c

DISCUSSION

Imported samples of purified papain gave low values on test and it is probable that the proteolytic activity had diminished on keeping Deterioration of samples of papain of Indian

manufacture whose dates of preparation are known, and which are kept in the laboratory at an average temperature of about 25°C, has been studied and the results are given in Table III The results show the extent of deterioration of papain samples of various grades of purity when kept at a temperature of about 25°C for periods of 3 to 6 months In every case a definite loss of activity has been observed, and the percentage loss has been found to increase with the increase in purity of the enzyme The dried juice appears to be more stable than the pure preparation The only explanation that can be offered for this interesting finding is that an impure preparation contains some substance which has the ability to protect the activity of the enzyme thus rendering it more stable, while in the process of purification of the enzyme, this protective substance is probably removed The nature of this protective substance is under investigation

Standard for papain and its preparations —The average activity of one gramme of papain manufactured in India, as determined by the assay method described in this paper, is equivalent to 36 c c of N/10 sodium hydroxide As it has been found that papain samples deteriorate on storage, an allowance for such loss should be made before fixing a rigid standard From the results of Table III we are justified in making an allowance of about 30 per cent as this is approximately the average loss in 3 to 6 months After making this allowance the average activity becomes 36 0 c c — 11 0 c c = 25 0 c c of N/10 NaOH This value represents a digestion of about 60 per cent of the substrate under the conditions described in the method of assay The same figure of 25 0 c c of N/10 NaOH can be taken as the standard for the papain preparations provided the specified quantities are added to the substrate in the assay method

Summary

The method of assay of papain activity described in the B P C does not give a true measure of the enzyme activity as the pH of the reaction mixture has been found to be 9 2 while the optimum pH for papain action is 5 0 to 7 0 As the standard is based on this method of assay, the 'standard' as well as the 'method of assay require modifications

A modified method of assay suitable for determining the activity of papain is described This method retains in broad principles the B P C method but the conditions of enzyme action and the quantities of substrate and enzyme suitable for assay have been changed

The rate of deterioration of samples of papain of various grades of purity, when kept at the temperature of about 25°C for periods of 3 to 6 months, has been studied It has been found that the cruder samples of papain retain their activity better than purer samples The nature of the protective substance in the crude samples of papain is under investigation

A standard for the activity of papain has been suggested, based on the average activity of papain samples manufactured in India and after making allowance of about 30 per cent for deterioration The standard recommended is —

'The amino acids produced by one gramme of papain in the assay process described in the paper should require not less than 25 0 c c of N/10 NaOH for neutralization'

The same standard of 25 0 c c of N/10 NaOH may be adopted for these following papain preparations —

1 Elixir papain B P C
2 Glycerinum papain B P C
3 Liquor-papain-*et*-iridine B P C

The quantities of each of these preparations to be added to the substrate in place of papain itself have been calculated and found to be —

1 5 c c adjusted to pH 5 and made up to 10 c c
2 5 6 c c adjusted to pH 5 and made up to 10 c c
3 13 5 c c adjusted to pH 5 and added to the substrate respectively

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Ind Jour Med Res 31, 2, October 1943

COMPARATIVE PHARMACOLOGY OF THE TOTAL ALKALOIDS OF *RAUWOLFIA SERPENTINA* BENTH OBTAINED FROM BENGAL BIHAR AND DEHRA DUN

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[Received for publication June 30 1943]

INTRODUCTION

THE root of *Rauwolfia serpentina* Benth mixed with pepper and made into a paste with water has been used as a cure for certain types of insanity for a long time in India Though so commonly used it is curious to note that this important virtue of the plant has not been mentioned in the classical books of the indigenous system of medicine The drug, mostly from the plant grown in Bihar has been in use more or less as a secret remedy Sen and Bose (1931) and Chopra Gupta and Mukherjee (1933) studied the pharmacological action of the plant and demonstrated the hypotensive property of the alkaloidal base extracted from the plant As a result of this preliminary work an alcoholic extract standardized to contain 0 5 per cent alkaloid has been prepared by the School of Tropical Medicine Calcutta This preparation has been extensively used by clinicians for the treatment of hyperpiesis and maniacal types of insanity The School extract is made from the plant grown in Dehra Dun as it was found to be pharmacologically more dependable than the extract made with the plants grown in Bihar The plant also grows extensively in Bengal The preference for the extract made from Dehra Dun plants by the clinicians for the treatment of hyperpiesis and the almost universal use of the Bihar samples as an insanity cure led us to investigate the comparative pharmacology of the root alkaloid of the species grown in different regions It is also known that the chemical constituents of the same species of a plant may vary not only quantitatively but also qualitatively according to the season of collection, stage of maturity of the plant and the region (soil and climate) of growth of the plant The chemistry of the roots of *Rauwolfia serpentina* Benth grown in Dehra Dun Bihar and Bengal is being worked out in the chemistry department of this School Investigations hitherto carried out have revealed differences both quantitative and qualitative in the important chemical constituents of these different varieties

EXPERIMENTAL METHODS

Preparation of the total alkaloid—The hydrochloride of the total alkaloid of the roots of *Rauwolfia serpentina* Benth used in these experiments was prepared in the department of chemistry of the School of Tropical Medicine

Two kilograms of the powdered drug were exhausted with rectified spirit The alcoholic extract was concentrated to semi-solid consistency and repeatedly extracted with water until free from alkaloid The water-insoluble residue was extracted with 1 per cent hydrochloric acid until free from alkaloid The acid and the watery extracts were mixed and freed from fatty substance with petroleum ether The fat-free extract was made alkaline and the alkaloidal base precipitated was extracted completely with chloroform Chloroform was distilled off and the residue containing the total alkaloids was dissolved in alcohol and neutralized with hydrochloric acid to form the hydrochloride of the total alkaloid from which the solvent was removed by evaporation *in vacuo* The hydrochloride of the total alkaloid thus obtained is a yellowish-brown mass soluble in water and alcohol

An aqueous solution usually of 1 per cent strength was used for the experiments The hydrochloride of the total alkaloid of the roots of *Rauwolfia serpentina*, Dehra Dun variety, forms a clear yellow solution, that from Bengal variety gives a semi-transparent yellowish-brown solution, while the alkaloidal hydrochloride from the Bihar plants gives a solution midway between the Dehra Dun and Bengal alkaloids in colour and transparency

Toxicity tests —Albino rats between 75 g to 125 g were used for the tests the calculated dose made up to 1 c c with normal saline was injected intraperitoneally before food in the morning

Toxic symptoms —In sublethal doses, the total alkaloids of all the three varieties cause a depression of the motor activity of the animals They tend to lie down quietly but can be roused to activity The sensory reception seems to be diminished Respiration is either deep and slow or shallow and frequent Death which usually supervenes 3 to 4 hours after the minimum lethal dose is due to respiratory failure, the heart goes on beating for some time after the respiration has stopped The post-mortem findings show dilatation of the heart and congestion of the brain Those animals which survive usually have an aversion to food for several days

Central nervous system

Motor cortex —Toads were used and the drug was introduced into the ventral lymph sac

Spinal cord (reflex activity) —(*i*) Cats decerebrated at the level of the pituitary stalk and also cats with the brain pithed and the spinal cord sectioned at the level of the second cervical vertebra were used The extent of knee jerk by uniform tapping on the patellar tendon and that of the withdrawal reflex (after pricking the food pad) were observed before and after test doses of the drug An interval of 1½ to 2 hours was allowed for recovery from the shock of operation before observations were made (*ii*) The reaction time of the reflex withdrawal of the foot on stimulation with dilute hydrochloric acid in pithed toads was measured

The hypnotic effect was observed in cats which received the drug in suitable doses by the stomach tube Observations on rats after intraperitoneal administration of the drug were also made

Circulation —Cats and rabbits under the influence of chloralose and urethane were used for blood pressure and myocardiographic experiments For localizing the site of action decerebrated and spinal cats were used Decerebration was done at the level of the hypothalamus under ether anæsthesia For spinal preparations, the spinal cord of cats under ether was sectioned at the level of the second cervical (in some experiments the fourth dorsal) vertebra, the spinal cord and brain anterior to the level of section was destroyed by pithing and plugging

In some of these experiments atropine was given to the animals to the point of producing absence of cardiac inhibition to vagal stimulation, while in other animals the sympathetic exciters were paralysed by ergotoxin (adrenalin causing a reversal of blood pressure)

Isolated heart —Kitten's heart perfused through the coronary arteries and toad's heart perfused through the inferior vena cava were used

Blood vessels —In some of the kymographic experiments, kidney, spleen and intestinal volume records were taken to find the effect of the drug on the local blood vessels

The time for the outflow of a given volume of perfusate and the outflow per minute were also noted in some toads with the brain and the spinal cord pithed The abdominal viscera were removed and the splanchnic vessels were ligatured in these experiments to exclude the action on the splanchnic vessels

Respiration —Decerebrated cats or cats anæsthetized with urethane were used the respiration was recorded either with tracheal tambour or by the diaphragmatic slip method

Intestines—In intact cats under chloralose anæsthesia the intestinal movements were recorded with Jackson's enterograph in some experiments both before and after atropine. For isolated organ experiments intestinal strips from kittens and guinea pigs were suspended in oxygenated Fleisch's solution in a Dale bath and the movements were recorded in the usual way.

Uterus—In intact animals under chloralose anæsthesia Barbour's technique was used. For isolated organ experiments strips of uterus of pregnant and of virgin guinea pigs were suspended in oxygenated Fleisch's solution in a Dale bath and the movements were recorded in the usual way.

Results

The results are presented in a tabular form for ready comparison and economy of space —

TABLE

	Rauwolfia serpentina (Dehra Dun) total alkaloid	*Rauwolfia serpentina* (Bihar) total alkaloid	*Rauwolfia serpentina* (Bengal), total alkaloid
M L d in albino rats	12·5 mg. per 100 g.	10 mg. per 100 g.	10 mg. per 100 g.
CENTRAL NERVOUS SYSTEM —			
(a) *Motor cortex*	Stimulant	Depressant	Depressant
(b) *Hypnotic effect*	Nil	Slight (in toxic doses)	More than Bihar alkaloid in toxic doses
(c) *Medullary centres* (vasomotor and respiratory)	Depressant	Depressant + +	Depressant +
(d) *Spinal reflexes*	Depressant	Depressant	Depressant

N B—The respiratory effect lasts longer than the circulatory effect. The frequency of respiration may be initially increased during the steep fall of B. P. presumably due to the carotid sinus reflex.

CIRCULATION —			
Cat under the influence of chloralose or urethane	B. P. falls, duration and extent less than the Bihar alkaloid	B. P. falls, duration and extent more than the Dehra Dun alkaloid	B. P. falls, duration and extent, the least of the three alkaloids

N B—The extent of fall is less after atropine. In spinal cats with the brain pithed and spinal cord sectioned at the level of the second cervical vertebra, all the three alkaloids cause a rise of B. P.

Heart	Depressed + +	Depressed + + +	Depressed +
Blood vessels of splanchnic area and limbs	Dilated	Dilated	Dilated
Bronchi	Initial constriction followed by dilatation	Initial constriction followed by dilatation	Initial constriction followed by dilatation
Uterus	Tone, contractility and rhythmicity increased	Tone, contractility and rhythmicity increased	Tone, contractility and rhythmicity increased

Ind Jour Med Res 31, 2 October 1943

ESTIMATION OF CHLOROCRESOL

BY

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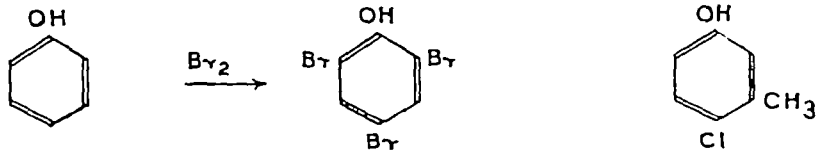
[Received for publication May 18 1943]

RECENTLY chlorocresol (para chloro meta cresol) has been included in the British Pharmacopœia (1932), Third Addendum and is being found to be a powerful germicide of low toxicity It is soluble in water (1 in 250) readily soluble in alcohol (95 per cent), ether and fixed oil It is volatile in steam and is being recommended as a bacteriostatic agent in preparations for administration by injection in a concentration of 0 1 per cent It would be of importance if a suitable method be now found for estimating chlorocresol in solution

In the determination of phenol Koppeschaar's method (1876) is invariably followed, it depends on the formation of a mixture of tribromophenol and tribromophenyl hypobromite by the addition of an excess of bromine the latter compound is easily decomposed on the addition of potassium iodide and the end product of the reaction is tribromophenol (I) The excess of bromine displaces an equivalent of iodine from the potassium iodide and may be titrated against standard sodium thiosulphate The bromine enters into the free ortho and para positions with the formation of tribromophenol It seems therefore that the quantity



(I) (II)

of bromine that might be taken up by a phenol molecule would depend upon the only free positions ortho or para to the phenolic hydroxyl group As such it would be quite reasonable to expect that the two ortho positions that are free in p-chloro-meta-cresol (II) would take up two bromine atoms by the Koppeschaar's method and this might be a measure in ascertaining the strength of the germicide in any solution

METHOD

Pure p-chlor-m-cresol (0 1042 g) m p 65° was accurately weighed into a measuring flask and it was brought into solution either by simply shaking with water or by adding about 10 c c of 0 1 N caustic soda solution and the volume was made up to 100 c c

About 2 c c of this solution was transferred to a stoppered-bottle and mixed up with 3 c c of potassium bromide solution (25 per cent), 10 c c of potassium bromate (about 2 8 per cent) and 10 c c of hydrochloric acid diluted with its own volume of water. The bottle was immediately stoppered and kept in the dark for 10 minutes with occasional shaking Then, two grammes of potassium iodide were carefully added so that no bromine vapour might escape, left aside for 10 minutes and then diluted to about 200 c c with water The liberated iodine was then titrated against 0 1 N sodium thiosulphate A blank experiment was also carried out side by side without the addition of any cresol solution

As cresol derivatives are known (*cf* Auwers, 1903, 1906) to undergo bromination at the side methyl group when treated with excess of bromine, so in the above estimation the reaction period was increased to 30 and 60 minutes The temperature of the reaction mixture was also varied The results obtained (Table I) indicate that chlorocresol only takes up the calculated amounts of bromine (i e two atoms) at the two free ortho positions to its hydroxyl

group In all these calculations 1 c c of 0 1 N sodium thiosulphate was taken as equivalent to 0 003565 g of chlorocresol The best result was obtained when the reaction was conducted at the room temperature (25°C) for a period of 30 minutes

TABLE I

Estimation of chlorocresol by bromination

Amount taken 10 42 mg

Figures indicate the amount found in mg

Reaction temperature	Period of reaction		
	10 mins	30 mins	60 mins
25°C	10 2	10 42	10 09
40°C	10 18	10 32	10 23

ESTIMATION IN SOLUTION

To find out whether the above method would be applicable when the preservative is present in small percentage such as in Injectio Quininæ et Urethani a 2-c c ampoule of the above medicament was taken for the quantitative estimation A preliminary experiment showed that the presence of quinine is not desirable, and as such quinine hydrochloride present was precipitated by addition of 6 c c of 2 N caustic soda solution It was filtered off and washed The filtrate and washings were carefully extracted with chloroform and finally acidified with hydrochloric acid The chlorocresol present was then estimated by the Koppeschaar's method The difference in result with a blank experiment indicated a strength of 0 11 per cent in average from three experiments, whereas the amount present in Injectio Quininæ et Urethani was 0 10 per cent

As chlorocresol is volatile in steam so an attempt was made to separate the same from a solution by steam distillation and estimate its strength from the distillate collected Distilling however 200 c c aqueous solution containing 10 42 mg chlorocresol by steam, it was noticed that about a litre of the distillate would have to be collected in order to make the mother solution free from chlorocresol , whence the strength of the preservative in the distillate dropped to about 1 042 mg per cent As such it was considered whether chlorocresol might be determined by some colorimetric means with the help of a ' phenol reagent (*cf* Chapin 1921)

ESTIMATION BY PHENOL REAGENT

Reagents —1 A standard chlorocresol solution

2 *Phenol reagent* —It is made (*cf* Folin and Ciocalteu, 1929 King and Armstrong 1934) by dissolving extra-pure sodium tungstate (10 g) and extra-pure sodium molybdate (2 5 g) in water (70 c c) It is mixed with 5 c c of syrupy phosphoric acid (85 per cent) and 10 c c concentrated hydrochloric acid The whole is refluxed for 10 hours , then lithium sulphate (15 g) and a few drops of bromine are added The reacted mixture is further boiled for 15 minutes, cooled and the volume made up to 100 c c For colorimetric estimation this stock solution is diluted with its own volume of water and is used as the ' phenol reagent

Method —0 75 c c of about 1 per cent solution of a standard chlorocresol and almost an equal amount of an unknown solution of chlorocresol were separately taken in two different 100 c c volumetric flasks To each, 2 c c of the phenol reagent were added This was followed by 2 c c of sodium carbonate solution (20 per cent) The volume in each flask was made up to 100 c c and the colour developed was compared in a Klett colorimeter immediately

Calculation —According to Beer's law upon which any colorimetric estimation is based the percentage of chlorocresol may be obtained by using the formula —

$$C_1 = \frac{R_2}{R_1} \times C_2 \times \frac{100}{V},$$

where C_1 Concentration of chlorocresol per 100 c c of the solution
C_2 Amount of standard chlorocresol used
R_1 Reading of the unknown
R_2 Reading of the standard chlorocresol
V Volume of the unknown solution taken

Estimation in water solution – A known amount of p chloro m cresol (Dr Frænkel and Dr Landau Berlin) m p 65°C was freshly dissolved in double-distilled water (pH ca 6 3) and was standardized by the bromide bromate method This solution was used as the standard chlorocresol in the following experiments The different amounts of this chlorocresol were again dissolved in the same quantity of water and these solutions were then treated with the phenol reagent as described above The colour developed was then respectively compared against the colour that was developed by the above standard chlorocresol solution The results are incorporated in Table II Each reading recorded is from average of three observations

TABLE II

Standard chlorocresol solution 0 1095 per cent,
Amount of standard solution taken 0 75 c c ,
C_2 0 00082125 g
Amount of unknown solution (V) 0 75 c c

Number of experiments	Unknown reading (R_1)	Standard reading (R)	Percentage of the unknown solution		Error	Percentage of error	Average percentage of error
			Found (C_1)	Taken			
1	16 7	20	0 1311	0 1314	0 0003	0 228	0 201
2	17 7	20	0 1237	0 1241	0 0004	0 322	
3	18 8	20	0 1165	0 1168	0 0003	0 257	
4	21 5	20	0 1019	0 1022	0 0003	0 294	
5	23 1	20	0 0950	0 0940	0 0001	0 105	
6	25 0	20	0 0876	0 0876	*Nil*	*Nil*	

ESTIMATION IN INJECTIO QUININÆ ET URETHANI.

One c c of the Injectio Quininæ et Urethani containing 0 1 per cent chlorocresol was taken in a 50-c c flask, and cautiously made alkaline with about 3 c c of 2 N caustic soda Quinine separated out and the volume was made up to 50 c c This was then filtered and the whole filtrate was extracted out with chloroform to remove any dissolved quinine The aqueous portion was separated out and 40 c c (equivalent to 0 8 c c of the original solution) of this was taken in a 100-c c volumetric flask, mixed with the phenol reagent and alkali as usual The colour developed was compared against a similar colour obtained from 0 75 c c of a 0 1042 per cent chlorocresol solution The experiment was repeated twice and the strength of chlorocresol in Injectio Quininæ et Urethani was ascertained from the formula indicated above

$C_2 = 0\ 0007815$ g , $R_2 = 20$, $V = 0\ 8$, $R_1 = 20$, 20 1 and 19 9
Therefore $C_1 = 0\ 0977$ 0 0972 and 0 09819 respectively
The average percentage of error is 2 3

ESTIMATION IN LIQUOR ADRENALINÆ HYDROCHLORIDE

Ten c c of the above solution containing 0 1042 per cent chlorocresol were diluted to 200 c c and distilled with steam One litre of the distillate was collected and 75 c c of the distillate (equivalent to 0 75 c c of the original solution) were taken for colorimetric estimation

as usual against 0 75 c c of a 0 1042 per cent chlorocresol solution The percentage of chlorocresol was found to be 0 1042—the standard reading synchronizing with the reading of the unknown

One c c of the above solution was again diluted to 20 c c and the chlorocresol was steam distilled until 100 c c were collected Seventy-five c c of this distillate (equivalent to 0 75 c c of the original solution) were taken and treated with the phenol reagent as usual The colour developed was compared against the colour developed by 0 75 c c of a 0 1042 per cent chlorocresol solution The reading of the standard (R_2) was 20 , reading of the chlorocresol solution from steam distillation (R_1) was 19 5 , whence the percentage of chlorocresol in Liquor Adrenalinæ was found to be 0 1069 from the formula as recorded previously The error that comes about is 2 6 per cent

Discussion

It is being found that p-chloro-m-cresol having the two free ortho positions with respect to the hydroxy (phenolic) group (*vide* formula II) reacts as usual with two atoms of bromine and as such this can be easily estimated by the customary bromide-bromate method The optimum period of reaction is being found to be 30 minutes at the room temperature The substance is not so easily soluble in water but the solubility can be increased by a slight addition of alkali Alcohol, however, should not be used in making a solution of chlorocresol as it interferes with the bromide-bromate titration The method can also be followed in estimating the preservative in Injectio Quininæ et Urethani and similar other preparations But the chlorocresol is to be separated out from the alkaloid and other interfering substances either by removing them by some solvent extraction, or separating out the chlorocresol from the mixture by steam distillation The strength of the preservative, however, lowers down to a considerable extent as such as an accuracy in estimation by chemical reaction may be questioned As phenolic bodies are known to undergo oxidation by ' phenolic ' reagent with the development of a distinct blue colour, advantage was taken of this reaction in finding a method of estimating the chlorocresol by colorimetric comparison In Table II it would be found that chlorocresol solutions differing in strength even by about ± 20 per cent from a standard chlorocresol solution are being found to follow the Beer's law and as such can be easily estimated by the ' phenol ' reagent and the average percentage of error does not exceed 0 201 It is, however being noticed that the maximum intensity of colour develops immediately after the addition of alkali In estimating the same in Injectio Quininæ et Urethani the error comes to about 2 3 per cent in the negative side As chlorocresol distills with steam, this may also be estimated in any solution like Liquor Adrenalinæ Hydrochloride For every milligram of the preservative present in the solution meant for estimation, 100 c c of the steam distillate is to be collected to ensure that all the amount present may be completely distilled off It may be noted that the amount of any solution to be used for reacting with the ' phenolic ' reagent should be more or less equivalent to the amount of chlorocresol present in the standard solution taken for colorimetric comparison

Conclusion

Chlorocresol can be estimated by bromide-bromate titration Alcohol alkaloids and similar other interfering substances must be removed before bromination

Chlorocresol may also be determined by the ' phenol ' reagent and the strength of the preservative in Injectio Quininæ et Urethani and Liquor Adrenalinæ Hydrochloride may be easily found out by separating the quinine from the former by solvent extraction and isolating the preservative from the latter by steam distillation

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Ind Jour Med Res 31, 2 October 1943

CHOLESTEROL AND HÆMOLYSIS

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To cholesterol and lecithin the most prominent representatives of the sterols and phosphatides respectively, some of the characteristic properties of cell membrane have been ascribed Though there is very little experimental proof indicating the specific function of these substances their great importance is indirectly inferred by their invariable presence wherever the phenomena of life are manifest According to Overton, the external limiting pellicle of the red blood corpuscles as in most living cells, is formed by a lecithin-cholesterol compound whose solvent power determines the permeability of the cell by foreign substances These substances also occupy a very prominent place in Bechhold's conception of the structure of stroma, which he regards as a protein mesh holding by adsorption a homogeneous mixture of lecithin and cholesterol According to him, hæmolysis is the result of loosening of this structure caused either by solution, swelling, precipitation or otherwise of any of the components of this complex colloidal mixture

Though cholesterol and lecithin are found so constantly in close association in the body, their mutual relation is one of antagonism in several important respects Cholesterol being a hydrophobic substance increases the resistance of the cell, while lecithin behaves quite the other way on account of its hydrophilic nature Kurten and Linzenmeir (quoted by Campbell 1924-25) have shown that cholesterol hastened while lecithin retarded sedimentation of the red cells This antagonistic relation is also supposed to be present with respect to hæmolytic phenomena Cholesterol and lecithin are said to have antagonistic and naturally neutralizing effect upon the hæmolysis of red cells by water, acids or alkalis (Degkwitz 1930) Lecithin is generally believed to accelerate hæmolysis and cholesterol to retard it It was formerly believed that lecithin activated not only venom hæmolysis but that a number of other substances of varied descriptions had the capacity to hæmolyse in combination with lecithin, even in those cases where the substances themselves in high concentrations did not hæmolyse (Friede, 1924) Roy and Chopra (1941), however, have shown that purified lecithin activates hæmolysis only in the case of snake venoms and a few other animal poisons and that in the large majority of instances, the so-called accelerating action of lecithin is due to free fatty acids or other impurities present in impure specimens of the substance and not due to any inherent property of lecithin itself

The anti-hæmolytic action of cholesterol was first demonstrated by Ransom (1901) who showed that it was able to prevent hæmolysis by saponin Stokes (quoted by Campbell, *loc cit*) found that a large amount of cholesterol in the blood protects rabbits from the usual hæmolytic effect of intravenous injection of saponin and it was believed that cholesterol retarded not only snake venom hæmolysis but that caused by a variety of other substances such as sodium oleate, optochin, quinine hydrochloride, carbolic acid, etc (Friede, *loc cit*) The resistance of the erythrocytes to saponin hæmolysis in disease was also found by Neilson and Wheelon (1921) to vary directly with the cholesterol content of the blood being decreased by phosphatides Their resistance to hypotonic saline solutions, however, has been shown by Delas (1933) to be independent of the cholesterol content of the corpuscles or plasma

In view of the rather wide and somewhat conflicting nature of the claims made with respect to the anti-hæmolytic action of cholesterol, it was considered desirable to repeat some of the experiments with a view to assessing its real significance with respect to some important types of hæmolysis

TABLE I

Saponin solution	0 05 per cent in normal saline
Cholesterol emulsion	1 in 1,000 , , ,
Sheep's r b c	3 per cent , ,

Number	R b c, c c	Saponin, c c	Normal saline, c c	S̄ CHOLESTEROL — Hæmolysis in				C̄ CHOLESTEROL 0 25 c c — R b c added last — Hæmolysis in				C̄ CHOLESTEROL 0 25 c c — Saponin added last — Hæmolysis in			
				½ hour	1 hour	2 hours	21 hours	½ hour	1 hour	2 hours	21 hours	½ hour	1 hour	2 hours	21 hours
1	0 3	0 5	0 2	+++++ in 7½ mm	+++++	+++++	++++	—	—	—	80 per cent	+++++ in 5 mins	+++++	++++ ±	+++++
2	0 3	0 2	0 5	—	—	—	20 per cent	—	—	—	—	—	—	—	—
3	0 3	0 1	0 6	—	—	—	—	—	—	—	—	—	—	—	—
4	0 3	—	0 7	—	—	—	—								
5	0 3	—	0 7					—	—	—	—				

+++++ = Complete hæmolysis
± = Doubtful hæmolysis
— = No hæmolysis

Though the anti haemolytic action of cholesterol was first brought out in connection with saponin hæmolysis the inhibitory action was subsequently reported to be very slight, comparatively larger doses being required to effect a retardation The real mechanism of saponin hæmolysis is not fully understood but it is believed that the saponins combine with cholesterol as well as with lecithin and once the affinities of a saponin have been satisfied by cholesterol it ceases to act upon the lecithin of the membrane of the red blood cells Consequently cholesterol prevents a saponin from causing hæmolysis and so acts as an antidote for saponin substances This theory postulates saponin hæmolysis as being due to the solvent action of saponin on the lecithin present in the membrane of the corpuscles

In the following experiments the action of cholesterol has been studied in the same general manner as in the case of lecithin (Roy and Chopra *loc cit*) (Tables I and II) —

TABLE II

Number	R b c, c c	Saponin c c	Normal saline c c	Cholesterol c c	S CHOLESTEROL Time for complete hæmolysis	C CHOLESTEROL Time for complete hæmolysis	REMARKS
1	0 3	0 5	0 2		1 -10"		
2	0 3	0 5	0 1	0 1		1 —30	In these experiments the r b c were added last after incubation of the other ingredients for one hour at 37°C
3	0 3	0 2	0 5	- -	30 —0"		
4	0 3	0·2	0 4	0 1		60 per cent in 45', ++++++ in 21 hours	
5	0 3	—	0 7		No hæmolysis in 21 hours		
6	0 3	—	0 6	0 1		No hæmolysis in 21 hours	

From Tables I and II it appears that cholesterol exerts a retarding action on saponin hæmolysis only when relatively big doses of the substance are employed and also when the r b c are added last that is to say when saponin and cholesterol are allowed to remain in contact for some time before the addition of r b c When, however, saponin is added last, there is no retardation In certain cases a slight acceleration is even observed This is probably due to the fact that saponin effects hæmolysis before it finds time to combine with cholesterol This also throws some light on the mechanism of the retarding action of cholesterol at least with respect to saponin hæmolysis

Experiments made with lecithin on parallel lines showed that it behaves in exactly similar manner towards saponin hæmolysis so that with respect to this type of hæmolysis these substances do not behave antagonistically

Cobra venom hæmolysis

It has already been pointed out that cholesterol is supposed to have a retarding action on cobra venom hæmolysis Our experiments in this respect are given in Table III —

Table III

Human r b c	3 per cent
Cobra venom solution	0 05 per cent
Cholesterol	1 in 1,000

Number	R b c, c c	Venom solution c c	Normal saline, c c	Cholesterol, c c	Hæmolysis in ½ hour	1 hour	2 hours	21 hours	Remarks
1	0 3	0 5	0 2		–	+++++	+++++	+++++	R b c added last after incubation of the other ingredients for ½ hour at 37°C
2	0 3	0 5	0 1	0 1	+++++	+++++	+++++	+++++	
3	0 3	0 05	0 65		–	–	20 per cent	45 per cent	
4	0 3	0 05	0 55	0 1	–	–	20 per cent	25 per cent	
5	0 3	0 5	0 2		+++++	+++++	+++++	+++++	Cobra venom solution added last after incubation of the other ingredients for ½ hour at 37°C
6	0 3	0 5	0 1	0 1	+++++	+++++	+++++	+++++	
7	0 3	0 05	0 65		–	–	+++++	+++++	
8	0 3	0 05	0 55	0 1	–	–	±	+++++	
9	0 3	0 4	0 3		–	+++++	+++++	+++++	In these experiments greater quantities of cholesterol were used without previous incubation of any of the ingredients
10	0 3	0 4		0 3	++	+++++	+++++	+++++	
11	0 3	0 2	0 5		–	+	+++++	+++++	
12	0 3	0 2	0 2	0 3	+++++	+++++	+++++	+++++	
13	0 3		0 7		–	–	–	–	
14	0 3		0 4	0 3	–	–	–	–	

These experiments show that cholesterol does not cause a significant retardation of cobra venom hæmolysis On the other hand, a slight acceleration is sometimes observed There are of course instances where a slight retardation is produced when the cholesterol solution is in contact with venom solution for a longer period than that stated above, but in no case does cholesterol retard venom hæmolysis to the extent that lecithin accelerates it

Bile salt hæmolysis

The relation of cholesterol to bile salt hæmolysis is of some interest in view of the close relationship of cholesterol and the cholic acid moiety of the bile salts and the probability that the latter is obtained entirely by the catabolism of cholesterol The literature on this subject however appears to be very scanty According to Bayer (1907) cholesterol has no retarding action on bile salt hæmolysis and lecithin produces inhibition but not in quantities that occur

in blood The following experiments will show that cholesterol retards bile salt hæmolysis in the same way as does lecithin (Table IV) —

TABLE IV

Sodium taurocholate (Difco)	0·5 per cent
Sheep's r b c	3 per cent
Cholesterol	1 in 1,000

Number	R b c c c	Na taurocholate c c	Normal saline, c c	Cholesterol c c	Time for complete hæmolysis	Remarks
1	0·3	0·5	0·2		0 – 20″	The other ingredients were mixed together and incubated at 37°C for ½ hour before r b c were added
2	0·3	0·5	0·1	0·1	1 – 0	
3	0·3	0·2	0·3		7 – 30″	
4	0·3	0·2	0·4	0·1	13 – 0″	
5	0·3	0·1	0·6		±	
6	0·3	0·1	0·5	0·1	–	
7	0·3	0·05	0·65		–	
8	0·3	0·05	0·55	0·1	–	in 21 hours
9	0·3		0·7			
10	0·3		0·6	0·1	–	

The observations of previous workers regarding the retarding action of lecithin on bile salt hæmolysis are also confirmed

SODIUM OLEATE HÆMOLYSIS

Experiments carried out on exactly similar lines show that cholesterol has no appreciable action on the hæmolysis caused by sodium oleate solutions

BACTERIAL HÆMOLYSIS

Under this head the action of cholesterol on vibrio (El Tor) hæmolysin and streptococcal hæmolysin was studied Vibrios were grown in one per cent peptone solution and *Streptococcus hæmolyticus* in serum broth Eighteen hours' old cultures were used in both the cases without centrifugation

Gohar (1932) showed that cholesterol had the effect of neutralizing the hæmolysin elaborated by the cholera vibrio The following experiments will show cholesterol has no appreciable action on vibrio hæmolysis (Table V) —

TABLE V

Number	Sheep's 3 per cent r b c, c c	Culture fluid c c	Normal saline, c c	Cholesterol 1—1,000, c c	Hæmolysis in			
					½ hour	1 hour	2 hours	21 hours
1	0·3	0·1	0·6		+ + + + +	+ + + + +	+ + + + +	+ + + + +
2	0·3	0·1	0·1	0·5	+ + + + +	+ + + + +	+ + + + +	+ + + + +
3	0·3	0·1	0·4	0·2	+ + + + +	+ + + + +	+ + + + +	+ + + + +
4	0·3	0·1	0·5	0·1	+ + + + +	+ + + + +	+ + + + +	+ + + + +
5	0·3	0·1	0·55	0·05	+ + + + +	+ + + + +	+ + + + +	+ + + + +
6	0·3		0·2	0·5	–	–	–	10 per cent
7	0·3		0·5	0·2	–	–	–	–
8	0·3		0·6	0·1	–	–	–	–
9	0·3		0·65	0·05	–	–	–	–

Experiments carried on with *Streptococcus hæmolyticus* on exactly similar lines showed that cholesterol was without any appreciable effect upon it In some of the experiments when cholesterol was left in contact with the lysin for some time before the r b c were added only a slight retardation was observed

Summary and conclusions

1 The action of cholesterol on some of the well-known hæmolytic agents, such as the saponins, cobra venom bile salts and bacterial hæmolysins has been studied

2 Cholesterol does not appear to have any considerable retarding action on hæmolysis as was formerly supposed

3 It retards saponin hæmolysis only when relatively big doses of the substance are employed and when saponin and cholesterol are allowed to remain in contact for some time before the r b c are added When, however, the saponin is added last there was no retardation whatsoever

4 It has no appreciable action on cobra venom hæmolysis nor on hæmolysis caused by sodium oleate

5 Cholesterol retards bile salt hæmolysis in the same way as does lecithin

6 It has no marked action on either the vibrio hæmolysin (El Tor) or streptococcal hæmolysin

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Ind Jour Med Res 31, 2 October 1943

INVESTIGATIONS OF GROUND WATER POLLUTION *

Part I

DETERMINATION OF THE DIRECTION AND THE VELOCITY OF FLOW OF GROUND WATER

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[Received for publication August 12 1943]

INTRODUCTION

The first phase of an investigation on ground water pollution naturally relates to the determination of the direction and the velocity of flow of ground water and thereby marks out the range for the experimental observation and also serves as a pilot-indicator for the pollution studies to follow It is now generally admitted that pollution does not travel in all directions from the place of its introduction but travels downstream with ground water and diffusion plays only a small part in the spread of pollution

Several methods have been suggested from time to time for the determination of the ground-water flow there is the mathematical method as developed by Hazen (1920) Slichter (1902 1905) Theim (1887) and Mark (1939), the laboratory method and lastly the field method Of these the last method involving direct measurement with the use of chemicals and bacteria as indicators gives by far the most accurate results as it is based on undisturbed samples of soil The method consists in placing a chemical in a central well and noting the time of its arrival in the surrounding wells either by electrical method or more simply by analysis of samples of water collected at suitable intervals In this connection Caldwell and Parr (1938) in their Alabama studies used sodium chloride as indicator with good results Previous work by the author (Dyer 1941) however showed that this chemical failed to indicate satisfactorily the flow in an alkaline alluvium in the Punjab.

The investigations on ground-water pollution initiated by the author (Dyer 1941*a*) have now been extended to other soil conditions obtaining in India The following experiments were undertaken to determine the flow of ground water as sodium chloride again was used for the purpose it is possible to check the Punjab results against a different soil

EXPERIMENTAL

Field experiments

Analysis of soil medium —The experiments described herein were carried out at Singur a rural area in West Bengal about 20 miles from Calcutta The area is typical of the plains of India and the Indo-Gangetic alluvium constituting them The soil of the locality is clayey silt down to a depth of about 16 feet Sand of medium size occurred at comparatively shallow depths below 16 feet The average composition of the soil obtaining at the site at different

* The studies and observations on which this paper is based were conducted with the support and under the auspices of the International Health Division of the Rockefeller Foundation

depths is presented in Table I The United States Bureau of Soils method was used in the determination of the amount of clay and silt present in samples

TABLE I

Mechanical analysis of soil

Depth in feet	FIGURES ARE MEANS OF 7 SAMPLES AT EACH DEPTH (EXPRESSED AS PER CENTAGE ON AIR DRY BASIS)		
	Sand	Silt	Clay
0 and 2	69 3	12 9	17 8
2 and 4	74 6	10 9	14 5
4 and 6	76 0	11 1	12 9
6 and 8	83 6	6 6	9 8
8 and 10	78 6	13 2	8 2
10 and 12	83 6	7 4	9 0
12 and 14	88 2	3 7	8 1
14 and 16	98 7	1 3	0 0
16 and 18	100 0	0 0	0 0
18 and 20	100 0	0 0	0 0

The figures clearly show that with increase in depth sand content increased and the amount of clay decreased At a depth of 14 feet there was a sudden change then clay was no longer present in the samples

The sand which is thus seen to occur below a 16-foot depth was analysed for those physical characteristics which might have a bearing on the rate of flow of ground water Mechanical analysis by the sieve method was used to find out the size and distribution of the sand grains The effective size and the uniformity coefficient of the samples at various depths were calculated by the graphical method and are presented in Table II The porosity ratio (percentage of void space to total volume) was determined and these figures are also included in Table II —

TABLE II

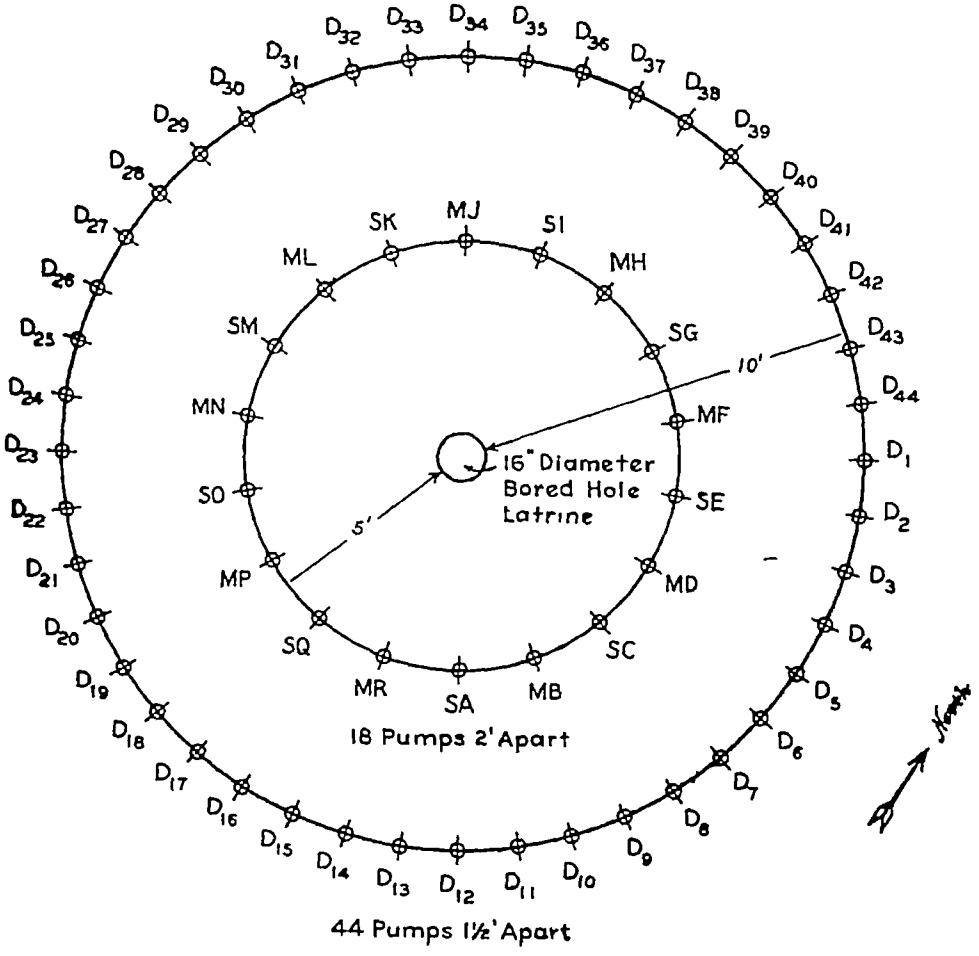
Physical characteristics of the soil in deeper layers

Depth in feet	AVERAGE OF 9 SAMPLES AT EACH DEPTH		
	Effective size	Uniformity coefficient	Porosity percentage
14 and 16	0 125 ± 0 03	3 0	44 1
16 and 18	0 170 ± 0 04	2 4	42 7
18 and 20	0 214 ± 0 03	1 9	41 1

The sand at the bottom layers was coarser and more homogeneous than at the top layers It may be seen from the results that the effective size uniformity coefficient and porosity were the order of about 0 2 2 0 and 10 per cent respectively which are within the limits specified by Allen Hazen for applying theoretical formulæ

Lay out of experimental field — After the nature of the soil medium had been determined, the field experiment was conducted to discover the velocity and direction of flow of the ground water as it actually existed The plan of the experimental field was similar to the one used by the author in his Punjab studies A central bore-hole (20 feet deep and 16 inches in diameter) was first installed as the central charging well The sides of the bore hole were protected from caving, in the first instance by a bamboo-matting and later on with a concrete casing, this casing was a 12-inch spun concrete pipe with $\frac{1}{2}$-inch holes spaced 6 inches centre to centre Two sets of wells encircling the central bore-hole were installed at distances of 5 feet and 10 feet respectively There were 18 wells in the 5-foot zone of alternating shallow (12 feet 3 inches) and medium (15 feet 3 inches) depth, spaced 2 feet apart In the 10 foot zone there were 14 deep (18 feet 3 inches) wells spaced $1\frac{1}{2}$ feet apart Schematic representation of the lay out is given in Graph 1 —

GRAPH 1

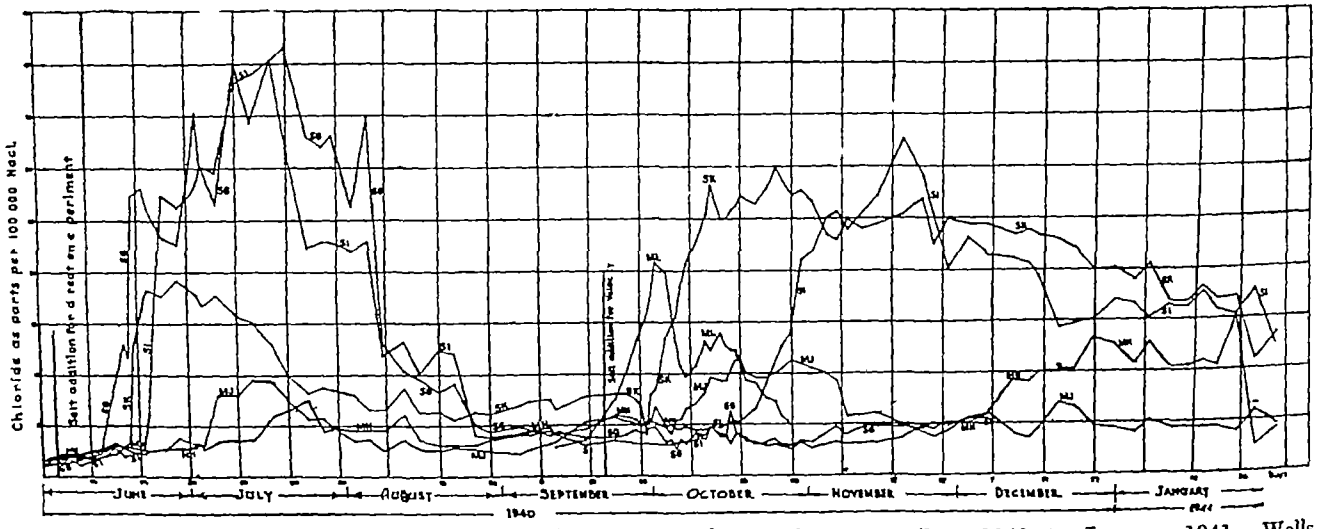


Arrangement of wells

Two experiments were carried out during the seasons June to September 1940 and September 1940 to January 1941 the first for the determination of the direction of flow and the second for the determination of the velocity of flow

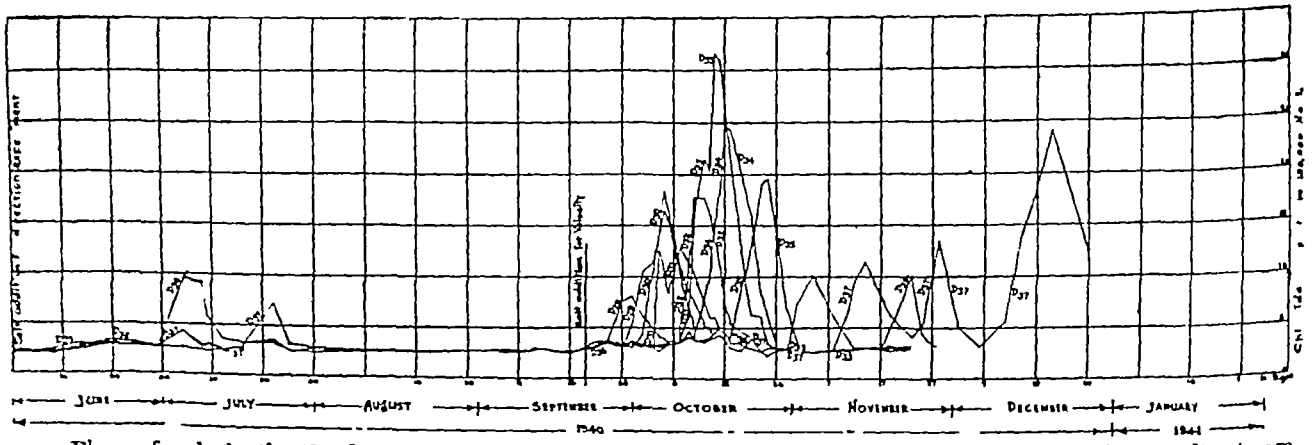
Direction of flow—The first experiment was started on 3rd June, 1940 and observations on the movement of salt were taken till the end of August Ten kilograms of salt were added to the central well, and the contents of the well were agitated for uniform distribution of the salt solution by diffusing air into the well at 50-pound pressure for a period of 20 minutes The ground water was tapped from the 5-foot and 10-foot zone wells by collecting samples daily to begin with and at stated intervals later on and these samples were tested for conductivity and chlorides The results are presented in Graphs 2 and 3 and Table III Chloride results only are presented, but conductivity determinations served as a useful check on chloride values there was close correlation between the two (Graph 5) Observations on water table and depth of the central bore-hole and rainfall during the period are presented in Graph 4

GRAPH 2



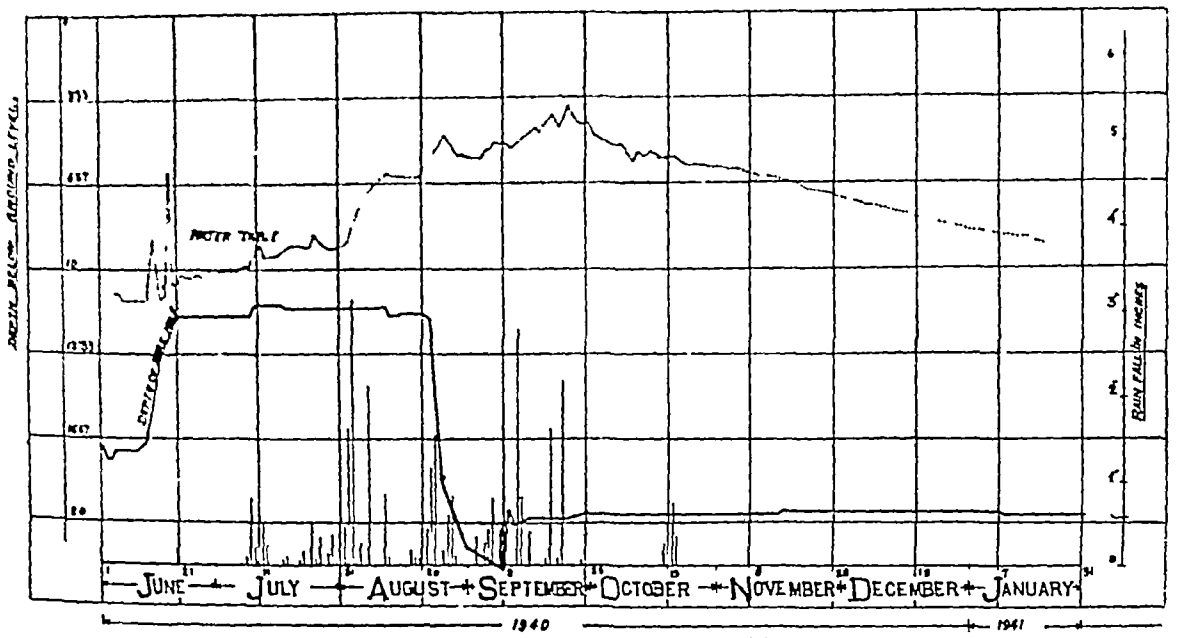
Flow of salt in the S, shallow, and M, medium, strata during the season June 1940 to January 1941 Wells in salt stream Shallow SG, SI, SK, and SM , Medium MH MJ and ML

GRAPH 3



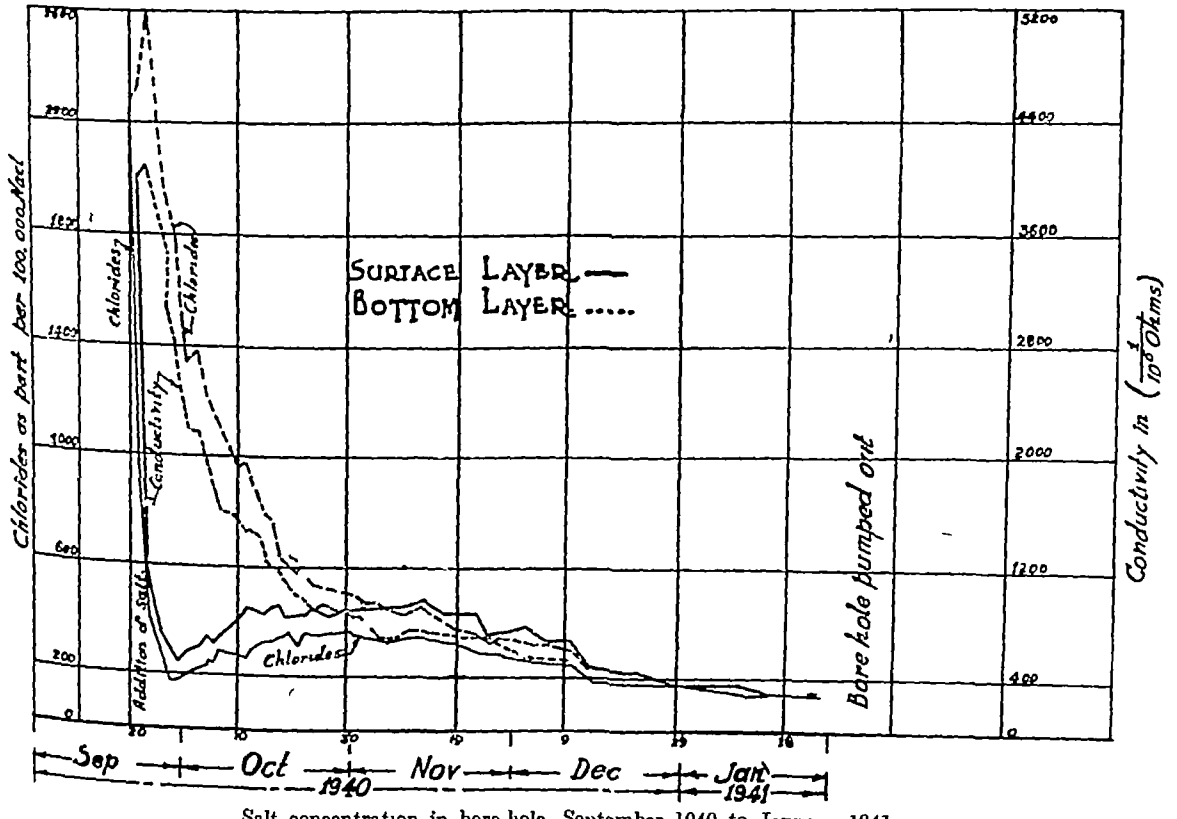
Flow of salt in the D deep, strata during the season June 1940 to January 1941 Wells in salt stream D28 to D37

GRAPH 4



Depth of water table rainfall and depth of bore hole

GRAPH 5



Salt concentration in bore-hole, September 1940 to January 1941

TABLE III

Weekly average salt content expressed as parts per hundred thousand NaCl

Weeks	CHLORIDE								
	SHALLOW			MEDIUM			DEEP		
	SO, SQ	SM, SK SI SG	SE, SC SA	MR, MP, MN	ML, MJ MH	MF, MD MB	D12 to D26	D27 to D40	D41 to D11
Before adding salt	1 24	1 28	1 53	1 96	1 79	1 68	2 25	2 29	2 23
1st Week after adding salt	1 70	1 68	1 67	2 21	2 20	2 03	2 45	2 33	2 43
2nd	2 25	3 15	2 74	2 43	2 78	2 54	2 89	2 82	2 84
3rd	3 86	13 78	6 91	2 19	3 71	3 00	2 87	2 85	2 88
4th	4 62	20 69	6 74	2 53	2 97	3 19	2 96	2 86	2 93
5th	4 24	22 08	6 88	2 62	4 33	3 26	2 84	4 18	2 83
6th	4 04	24 60	7 43	2 56	6 44	2 94	2 82	4 69	2 90
7th	3 59	23 39	10 20	2 67	5 97	3 28	3 24	3 46	3 14
8th	2 83	17 29	6 00	2 33	4 53	4 25	2 71	2 98	2 86
9th	2 85	16 20	5 42	2 37	3 55	4 18	2 80	2 89	2 84

The results presented above show that significant movement of salt took place in the shallow stratum only Salt appeared in a remarkably large proportion of wells, namely, 6 out of 9 shallow wells, in the 5-foot zone At the end of 30 days, however, a narrow salt stream was detected in the deep and medium strata also, but this stream persisted only for a few days The centre line of flow in all the three strata lay along well MJ

After addition of salt in the bore-hole on the 3rd of June, there was no reaction in any of the wells until the 17th June As the days were rainy and the water table had begun to rise, the bamboo basket which had been placed in the bore-hole to prevent caving was removed on the 17th June to be replaced by concrete piping This removal evidently disturbed the sand in the neighbourhood of the bore-hole, for one may see from the graph that the bore-hole caved in immediately The depth of the hole decreased from 17 feet to 11 feet below ground-level (Graph 4) Instantaneously salt appeared in the well SG It was a sudden movement, the salt content rose from 2 5 to 13 parts per hundred thousand A few days later, wells SK and SI also showed large increases in salt which persisted for some time, but the medium and deep wells did not show such conspicuous changes The results are briefly presented in Table IV —

TABLE IV

Recovery of salt in different wells

Strata well	Date of first appearance of salt	Duration	Maximum amount of salt in p p h t * NaCl
Shallow SG	June 17	(Indefinite—more than one month)	41 12
, SM	, 17	,	22 35
, SK	,, 19	,	19 31
SI	,, 22		40 26
SC	,, 17	1 month	12 87
SE	, 21	6 weeks	13 86

* p p h t = parts per hundred thousand

TABLE IV—*contd*

Strata well	Date of first appearance of salt	Duration	Maximum amount of salt in p p h t * NaCl
Medium MB	June 22	4 days	6 44
MI	July 6	11	7 10
MI	6	1 month	9 57
Deep D34	July 4	12 days	9 41
D35	4	18	17 74
D36	4	8	9 57

* p p h t = parts per hundred thousand

The direction of flow indicated by the results seems to be fairly correct as judged from the wells at all depths A possible explanation for the width of the stream may be that caving had loosened the texture of the sand in the vicinity of the bore hole and in consequence led to a more diffuse flow of the salt stream This explanation is supported by the fact that salt began to flow in the wells only after the heavy caving of sand It was observed at a later stage that the surface soil in that area had sunk down an effect which again may have been due to loosening of the soil texture in that region The explanation for little recovery of salt in the deeper layers is also fairly simple After caving had occurred, the depth of the bore-hole was not even 12 feet as the medium and deep wells were 15 feet and 18 feet respectively, below ground-level, not much salt could possibly flow to them

Velocity of flow—When the salt stream had dropped down to normal, the central bore-hole was again re bored to 19 feet and charged with 10 more kilograms of salt on 21st September, 1940, for the velocity experiment Observations on the flow of salt were continued till January 1941 with the same procedure as in the previous experiment The results obtained are included in Graphs 2 and 3 and Table V Water table, latrine depth and rainfall during the period are also included in Graph 4 There was no caving throughout this experiment

The velocity experiment was conducted under somewhat different conditions from those of the previous experiment and the soil in the bored area appeared to have attained a state of equilibrium But the period of experiment was during the middle of the monsoon, marked by heavy rainfall and a high water table which reached a maximum of 3 feet below the ground-level during the early part of the experiment (Graph 4) The water table decreased steadily after reaching the maximum of 3 feet

TABLE V

Weekly average salt content expressed as parts per hundred thousand NaCl

Weeks	CHLORIDE								
	SHALLOW			MEDIUM			DEEP		
	SO SQ	SM, SK, SI, SG	SE, SC, SA	MR, MP, MN	ML, MJ, MH	MF, MD, MB	D12 to D26	D27 to D40	D41 to D11
Before adding salt	1 24	1 28	1 53	1 96	1 79	1 68	2 25	2 29	2 23
1st week after adding salt	2 81	4 92	3 34	5 60	7 35	3 38	3 07	3 36	3 37
2nd "	2 77	5 60	2 70	4 52	9 17	2 93	3 18	4 42	3 16
3rd "	3 00	9 84	3 43	3 68	7 77	3 53	3 41	5 70	
4th "	2 81	10 55	3 18	3 29	8 60	3 08	3 55	6 94	

Salt recovery in the different wells is summarized in Table VI —

TABLE VI

Salt recovery from wells

Strata	Well	Date of first reaction	Date of maximum reaction	Date of fall in salt	Maximum amount of salt
Shallow	SK	Sept 30	Oct 12	*	28 22
,,	SI	Oct 19	Nov 11	*	27 39
Medium	ML	Sept 23	Oct 1	Nov 1	20 87
,,	MJ	Oct 6	,, 18	,, 11	11 22
,,	MH	Dec 23	Dec 27	Jan 29	13 40
Deep	D28	Sept 27	Sept 30	Oct 5	8 09
,,	D29	,, 30	Oct 6	,, 14	16 34
,,	D30	Oct 4	,, 7	,, 16	15 67
,,	D31	,, 5	,, 9	,, 18	12 87
,,	D32	,, 11	,, 15	,, 23	17 66
,,	D33	,, 11	,, 16	,, 26	31 52
,,	D34	,, 12	,, 18	,, 28	24 26
,,	D35	,, 23	,, 26	Nov 1	19 64
,,	D36	Nov 1	Nov 4	,, 11	9 98
,,	D37	,, 11	Jan 11	*	30 36
,,	D38	,, 25	,, 25	*	15 34

* Salt in these wells persisted till the last day of observation, 31st January, 1941

From the foregoing results it may be concluded that the direction of flow from the initial line along the wells ML and D28 changed during this period and swung towards the right When the results of the first experiment and the alteration in the water table had been taken into consideration the normal direction of flow appeared to be along the wells SG to SM in the 5-foot zone and D34, D35 and D36 in the 10-foot zone, although during the monsoon the centre line of flow turned through an angle of about 45 degrees along wells ML and D28, but later on ended rapidly to return to its original position

A remarkable feature of the second experiment was the rise and fall of the salt content of the wells one after another in succession (Graphs 2 and 3, and Table IV) This was observed in both the 5-foot and 10-foot zone wells, however, the salt appearance in the successive deep wells was periodic and quite characteristic of the swing of the salt stream

Actually, salt appeared in the well ML within two days after the addition of salt to the bore-hole, giving a velocity of more than $2\frac{1}{2}$ feet per day, and in the well D28 within 6 days, giving a velocity of more than $1\frac{1}{2}$ feet per day But as the salt did not arrive at the 10-foot zone till after 4 days, $2\frac{1}{2}$ feet per day seem to be the maximum

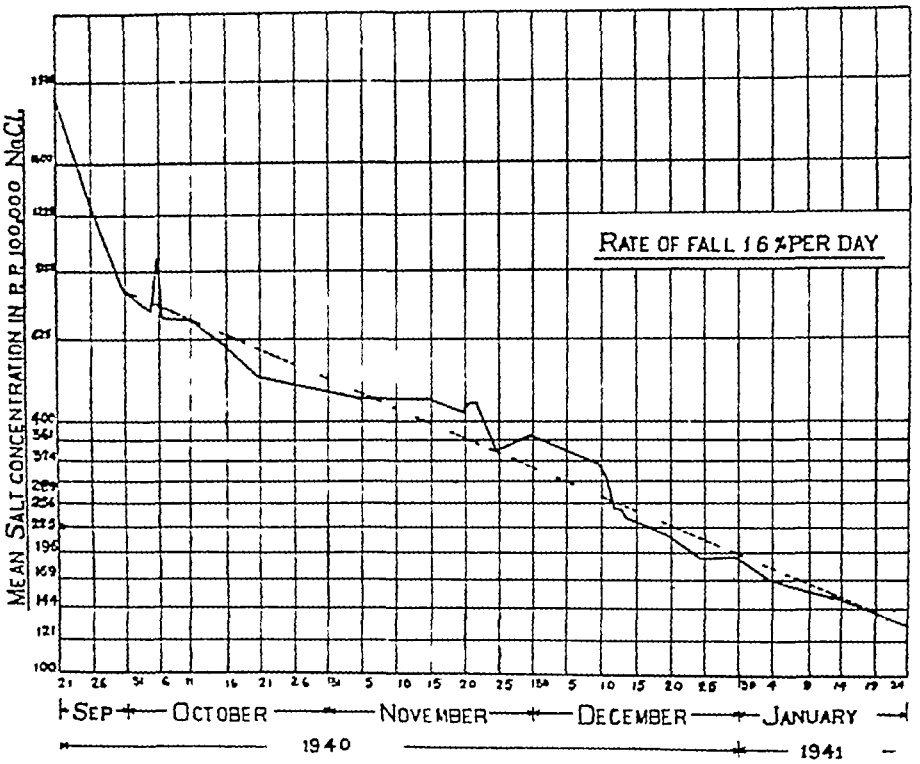
It would appear that during the monsoon the direction of ground-water flow changes, and judging by the rapidity with which salt appeared in the medium and deep wells, the velocity may be as high as $2\frac{1}{2}$ feet per day The difference between the velocities in the D and M strata is not very significant, although in the S stratum the velocity may be less than in the other two strata

Movement of salt in the central bore-hole —The change in the salt concentration in the charging well was followed throughout the period of experiment with a view to seeing how it flowed out of the bore-hole Surface and bottom samples were examined daily for conductivity and chlorides As caving interfered with the salt concentration in the first experiment, the results of the second experiment only are given in Graph 5

The results show that the surface layers were becoming diluted more rapidly than the bottom layers Probably salt was diffusing at the bottom into the ground water, while fresh water was flowing in at the surface layers diluting the contents at the top

The mean concentration of salt in the bore hole is presented in the chart in Graph 6 —

GRAPH 6



Mean salt concentration in the bore hole, September 1940 to January 1941

It is clear from the figure that salt concentration throughout the period shows a constant proportionate decrease An attempt was made to calculate the volume of outflow from the bore-hole, which information will no doubt be of use in the pollution studies to follow

If v be the volume of water in the bore-hole and p the concentration of salt at any time, and x be the volume of water which flows in, then the relationship between x, v and p can be expressed as follows —

$$\frac{x}{v} = \frac{1}{p} \times \frac{dp}{dt}$$

From Graph 6 it is calculated $\frac{1}{p} \times \frac{dp}{dt}$ is constant and equal to 1 6 per cent per day Therefore, the volume of water which flows is about 1/60 of the total volume of water in the bore-hole In the present experiment, when the water table remained at 8 feet almost a gallon of water per day flowed in and out of the bore-hole The calculation of the linear velocity is, however, not possible owing to the presence of the concrete casing inside the bore-hole

Laboratory experiments

Transmission constant of sand samples —Experiments were conducted in the laboratory to measure the transmission constant, or the coefficient of permeability (k) A sufficiently large number of representative soil samples, collected from the experimental sites, were used

for the purpose The method adopted at the Irrigation Research Institute, Punjab (Vaidhnathan and Luthra, 1934), was used for this determination The results obtained are given in Table VII —

TABLE VII

Transmission constant of sand samples

Depth in feet	Transmission constant (cm per sec)								
	1	2	3	4	5	6	7	8	9
14 to 16	0 052						0 062		0 040
16 to 18	0 064	0 081	0 041	0 063	0 042	0 033	0 061	0 007	0 057
18 to 20	0 096	0 107	0 096	0 090	0 102	0 043	0 031	0 071	0 055

According to Hazen's formula, the transmission constant is proportional to the square of the effective size

An attempt is made, therefore, to correlate the transmission constant with the known effective size for each sample The data are presented in Table VIII and Graph 7 —

TABLE VIII

Correlation of transmission constant with effective size of sand samples

(Eff size) (in mm)	Transmission constant (ft per day)	(Eff size)^2 (in mm)	Transmission constant (ft per day)	(Eff size) (in mm)	Transmission constant (ft per day)
0 0225	148	0 0225	179	0 0576	174
0 0361	181	0 0625	256	0 0324	88
0 0400	271	0 0289	120	0 0256	20
0 0361	230	0 0625	289	0 0361	200
0 0484	304	0 0196	94	0 0256	139
0 0121	116	0 0256	121	0 0400	162
0 0576	271	0 0361	175	0 0400	156

There is a striking correlation, and the regression of transmission constant (k) on square of effective size (d^2) is found to be 3,700 (Graph 7) We thus get Transmission constant in feet per day $= 3{,}700$ (effective size)$^2 + 40$

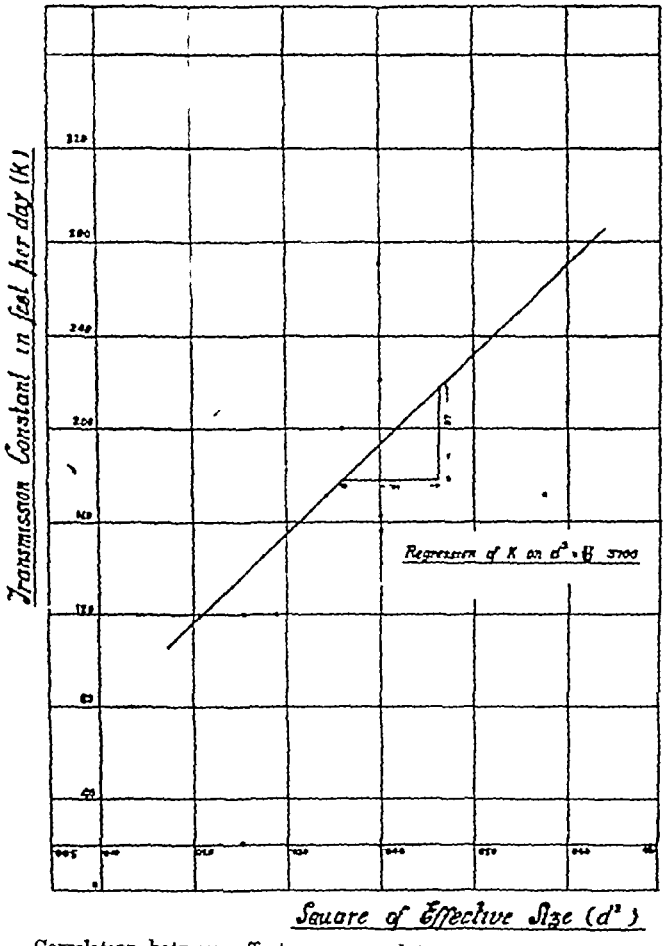
Hazen's formula is $v = C\, d^2 \frac{(t+10)}{60} \frac{h}{l}$ where v is the velocity, C is a constant $= 2{,}300$, t is the temperature Fahrenheit and $\frac{h}{l}$ is the slope For a temperature 80°F, which was the temperature of the ground water, the transmission constant will be $d^2 \times 2{,}300 \frac{(80+10)}{60}$ $= 3\,450 \times d^2$ This is in close agreement with the experimental value

Correlation between theoretical and observed velocity—Transmission constants and porosities of the sand samples collected between the central bore-hole and the 10-foot zone are made use

of m c l c u l a t i n g the theoretical v e l o c i t y by Darcy s formula According to Darcy s formula the field velocity = $\frac{\text{Transmission constant}}{\text{Porosity}} \times \text{slope}$

Since no slope measurements were made during the year (as the observation wells were installed only after the velocity experiment) the data for the corresponding period of the following year are made use of in this connection Monsoon conditions during these two years were quite comparable and it was therefore considered that calculations on this basis would give at least an approximate value for the velocity Average transmission constant and porosity for M strata were found to be 201 feet per day and 41 9 per cent respectively and for

GRAPH 7



Correlation between effective size and transmission constant

D strata 286 feet per day and 41 7 per cent Slope observed was 1/269 and the centre line of direction of flow swung through an angle of 45° so that the real slope is 1/269 × sec 45° = 1/186 Velocity in M strata is therefore $201 \times \frac{1}{186} \times \frac{1}{41.9} = 2.6$ feet per day The corresponding value for the D strata will be 3 7 feet per day

Average velocity in the D and M strata is of the order of 3 feet per day It may be observed that this value is comparable with actual velocity obtained in the field from the salt data, namely, 2 5 feet per day

Discussion

The most important observation arising out of the present work is that the direction of flow of ground water, as indicated by the movement of salt introduced into it, was more or less unchanged during the period June 1940 to January 1941 This period corresponds with the maximum fluctuations in the water table during the year when the ground-water flow is also susceptible to great variations It may, therefore, be considered that the data on flow of salt recorded in this paper are a fairly adequate indicator of the ground-water flow at the experimental site The abnormal condition of change in direction which persisted for a very short period during monsoon will also have to be taken into account in this connection

The laboratory experiments showed that there is close correlation between the transmission constants of samples in the sand region and velocity as determined by the field experiment With the slope known during any season, it may, therefore, be possible to predict the velocity of flow in that season from the transmission constant and porosity data This information will indeed be very useful in judging the rapidity of flow of water in the different zones and during different seasons in the year

It has also been shown that change in salt concentration in the central bore-hole can be made use of in determining the outflow of effluent from the bore-hole under normal conditions Although it is difficult to estimate accurately the linear velocity from these data, it may still be useful as a comparative measure of the velocity, especially in clayey soil medium where the velocities are too low to be determined by salt recovery in the wells

It would appear from the findings recorded in this paper that the use of salt as indicator of ground-water flow has no inherent defect in an acid or neutral soil Its partial failure at the Punjab sites (Dyer, 1941*a*), where the soil was a typical alkaline alluvium, may have been due to the fact that the natural flow of salt along with the ground water in these strata was too slow to be detected at 5 feet and 10 feet distance during the period of the experiment However, when acid was added during the experiment it may have reacted with the clay in addition to the 'kankar' (lumps of impure calcium carbonate common in the soil of that region of the Punjab) This reaction may have changed the colloidal nature of the clay and loosened the texture of sand, thereby resulting in more rapid flow

A fifth observation is that in studies on ground-water flow, field experiments using chemicals as indicators alone are inadequate unless they are supplemented by slope measurements A few observation wells sunk at different points at the experimental site would serve the purpose These data will also be useful on the computation of velocity based on the transmission constant and other physical properties of the soil samples obtaining in the field

Summary

1 The soil at the experimental site is clayey silt down to 16 feet with sand of medium size below this region The physical characteristics of the sand were within limits specified by Hazen for applying theoretical formulæ

2 Experiments using sodium chloride as indicator for determining direction of flow of ground water are described Salt was added to the bore-hole on 3rd June, 1940, and observations on the movement of salt taken till 31st August, 1940 Because of the caving of sand in the central bore-hole there was conspicuous movement of salt in the shallow strata only The direction of flow in all the three strata lay along well MJ

3 An experiment for determination of velocity is next described It was carried out during the season from September 1940 to January 1941, when there was great fluctuation in the water table and slope No caving occurred during this period and salt flowed freely through all strata During the height of monsoon the direction of flow swung towards one side momentarily but soon returned to the original direction The direction of flow of ground water was more or less the same in both experiments

4 Judging from the rapidity with which salt appeared in the medium and deep wells the velocity of flow during the period might have been as high as $2\frac{1}{2}$ feet per day, there was no significant difference in the velocities in the D and M strata but in the S stratum the velocity was less than those in the other two strata

5 Salt flowed from the charging well downwards and to a less extent laterally while water was flowing into the well diluting the contents at the surface, there was constant proportionate decrease in the salt concentration in the bore hole It was estimated that when the water table was 8 feet about one gallon of water per day flowed out of the latrine

6 There is close correlation between transmission constant of sand samples and the velocity of flow as determined by the field experiment

7 The significance of the foregoing observations in interpreting the flow of pollution is discussed

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G

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K

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